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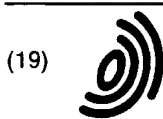
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Hefe-Promoter

Promoteur de levure

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EP 0 424 117 B1

Description

The present invention relates to yeast promoters, in other words nucleotide sequences which will direct expression of coding sequences in yeasts, for example *Saccharomyces cerevisiae*. Several such promoters have previously been isolated from yeasts and shown to be useful for directing the expression of heterologous coding sequences in yeast. The term "heterologous" in this specification is used to mean that the coding sequence is not the one whose expression is directed by the promoter in the wild-type organism in which the promoter is found; usually the coding sequence is one which is not found in the wild-type organism at all.

Sprague *et al* (1977 *J. Bact.* **129**, 1335-1342) disclosed yeast mutants deficient in the *GUT2* gene, which encodes a glycerol-3-phosphate dehydrogenase. Chen (1988 *Diss. Abstracts Int. B* **49**(3), 723) disclosed the purification and characterisation of a yeast glycerol-3-phosphate dehydrogenase. Kingsman *et al* (1990 *Methods in Enzymol.* **185**, 329-341) disclosed various promoters useful in yeast, including that of phosphoglycerate-kinase.

We have now found a further yeast promoter which can be used in this way, as can fragments of it, with advantages which were not predictable.

One aspect of the invention provides a DNA promoter sequence SEQ1, or a variant or a functional portion of said sequence, in isolation from the coding sequence which would normally neighbour the said sequence in wild-type *Saccharomyces cerevisiae*.

Although it will usually be undesirable, because a fusion protein will be produced when the promoter is used to express a heterologous protein, the promoter of the invention may be accompanied by a portion of the coding sequence which normally abuts it.

A "variant or functional portion" of the sequence is one which has minor variations of nucleotides and/or a shorter length, respectively, but which still retains at least 80% (preferably 90%, 95% or 99%) of the ability of the said sequence to promote transcription of a human albumin coding sequence positioned downstream thereof, with the other parameters of the two expression systems which are being compared (such as 3' regulatory regions) being the same. Alternatively, the variant or portion need retain only 10% of the said transcription-promoting activity provided that it is repressed by complex carbon sources and derepressed by the absence of such sources. In the case of a portion of the said sequence, such regulatory activity may be determined for the portion alone (ie without any other 5' regulatory sequence) or in conjunction with another 5' regulatory sequence positioned 5' or 3' to the said portion. A "variant" has 80%, preferably 90%, 95%, or 99% homology with the said sequence.

A "functional portion" of the sequence has 80%, or, preferably, 90%, 95%, 99% or 100% homology with the most homologous region of the said sequence. The portion is at least 100 nucleotides long, more preferably at least 200, 300, 400, 500, 1000 or 1500 nucleotides long. Suitably, the "functional portion" retains the ability of the said sequence to be repressed in the presence of complex carbon sources such as glucose and sucrose and to be derepressed in the absence of such sources whether or not glycerol or ethanol are present.

Suitably, the 3' end of any "functional portion" corresponds to the 3' end of SEQ1 and the said portion extends continuously away from the said 3' end in a 5' direction for up to about 1.35 or 1.40 kbp, beyond which (in the native environment) there appears to be a gene for Ala-tRNA^{GCU}. In nature, the 3' end of SEQ1 immediately precedes the ATG start codon.

Advantageously, the functional portion comprises SEQ3, in other words the 379 bp region immediately upstream of the ATG start codon, optionally with further 5' sections of the said sequence.

SEQ1, variants, portions and arrangements thereof described above are hereinafter referred to as a promoter of the invention.

The glycerol-3-phosphate dehydrogenase coding region has been shown to be homologous to the glycerol-3-phosphate dehydrogenase genes of mouse, rabbit, and *Drosophila melanogaster* with which it has 64, 60 and 56 percent homology respectively. Glycerol-3-phosphate dehydrogenase is one of two enzymes required to convert glycerol into dihydroxyacetone phosphate; these are essential genes if glycerol is supplied as a sole carbon source. SEQ2 shows a part of the 5' region flanking the promoter and is constituted by the SEQ1 region plus 123 bp upstream thereof, and the ATG start codon.

A promoter of the invention may be located on a cloning vector or an expression vector adjacent a restriction site such that a heterologous coding sequence may be located downstream of the promoter and in correct reading frame in relation to a translational start codon. The start codon may be provided on the vector (eg immediately 3' to the promoter) or it may be inserted as a 5' end of the heterologous coding sequence. A linker may be provided between the promoter of the invention and the start codon, if desired. 3' regulatory regions may similarly be provided on the vector or inserted with the coding sequence. The transcription termination signal is preferably the 3' flanking sequence of a eukaryotic gene which contains proper signals for transcription termination and polyadenylation in fungi. Suitable 3' flanking sequences may, for example, be those of the *GUT2* gene or they may be different, preferably the termination signal is that of the *S. cerevisiae* *PGK1* or *ADH1* genes. Preferably, the DNA construct according to the present invention is provided at both ends with synthetic oligonucleotide linkers which allow insertion and cloning of the construct in a

cloning vector. The promoter of the invention, the DNA coding sequence and the fungal transcription termination signals are operably linked to each other, ie they are juxtaposed in such a manner that their normal functions are maintained. Thus, the array is such that the expression control sequence effects proper expression of the coding sequence and the transcription termination signals effect proper termination of transcription and polyadenylation. The junction of these sequences is preferably effected by means of synthetic oligonucleotide linkers which may carry the recognition sequence of an endonuclease.

According to the present invention there is further provided a vector having one or multiple DNA inserts each comprising a promoter of the invention, a DNA segment consisting of a DNA sequence coding for a desired polypeptide which DNA segment is under transcriptional control of said promoter, and a DNA sequence containing eukaryotic transcription termination signals.

The vectors according to the invention are plasmids or linear DNA vectors and are selected depending on the host organism envisaged for transformation.

The invention relates also especially to plasmids which apart from the expression control sequence, the above DNA segment and the sequence containing transcription termination signals contain additional DNA sequences which are inessential or less important for the function of the promoter, ie for the expression of the desired polypeptide, but which perform important functions, for example in the propagation of the cells transformed with said plasmids. The additional DNA sequences may be derived from prokaryotic and/or eukaryotic cells and may include chromosomal and/or extra-chromosomal DNA sequences. For example, the additional DNA sequences may stem from (or consist of) plasmid DNA, such as bacterial or eukaryotic plasmid DNA, viral DNA and/or chromosomal DNA, such as bacterial, yeast or higher eukaryotic chromosomal DNA. Preferred plasmids contain additional DNA sequences derived from bacterial plasmids, especially *Escherichia coli* plasmid pBR322 or related plasmids, bacteriophage, yeast 2 μ plasmid, and/or yeast chromosomal DNA.

In the preferred plasmids according to the invention, the additional DNA sequences carry a yeast replication origin and a selective genetic marker for yeast. Plasmids containing a yeast replication origin, eg an autonomously replicating segment (ars), are extrachromosomally maintained within the yeast cells after transformation and are autonomously replicated upon mitosis. Plasmids containing sequences homologous to yeast 2 μ plasmid DNA can be used as well. These plasmids may be integrated by recombination into 2 μ plasmids already present within the cell or may replicate autonomously. The integration vectors of EP-A-251 744 or the "disintegration" vectors of EP-A-286 424 may be used.

As to the selective gene marker for yeast, any marker gene can be used which facilitates the selection for transformants due to the phenotypic expression of the marker. Suitable markers for yeast are particularly those expressing antibiotic resistance or, in the case of auxotrophic yeast mutants, genes which complement host lesions. Corresponding genes confer, for example, resistance to the antibiotic cycloheximide or provide for prototrophy in an auxotrophic yeast mutant, for example the *URA1*, *URA3*, *ARG4*, *LEU2*, *HIS4*, *HIS3*, *TRP5* or *TRP1* gene.

Advantageously, the additional DNA sequences which are present in the plasmids according to the invention also include a replication origin and a selective genetic marker for a bacterial host, especially *Escherichia coli*. There are useful features which are associated with the presence of an *E. coli* replication origin and an *E. coli* marker in a yeast hybrid plasmid. Firstly, large amounts of plasmid DNA can be obtained by growth and amplification in *E. coli* and, secondly, the construction of plasmids is conveniently done in *E. coli* making use of the whole repertoire of cloning technology based on *E. coli*. *E. coli* plasmids, such as pBR322 and the like, contain both *E. coli* replication origin and *E. coli* genetic markers conferring resistance to antibiotics, for example tetracycline and ampicillin, and are advantageously employed as part of the yeast vectors.

The vectors according to the invention may contain one or multiple DNA inserts each comprising *inter alia* the expression control sequence and the DNA sequence encoding the desired protein. If the vectors contain multiple DNA inserts, for example 2 to 4 DNA inserts, these can be present in a tandem array or at different locations of the vector. Preferred vectors contain one DNA insert or DNA inserts in a tandem array. The DNA inserts are especially head to tail arranged.

The plasmids according to the invention are prepared by methods known in the art. The process for the preparation of the vectors comprises introducing one or multiple DNA constructs containing a promoter of the invention, a DNA segment consisting of a DNA sequence coding for a desired polypeptide which DNA segment is under transcriptional control of said expression control sequence, and a DNA sequence containing fungal transcription termination signals, as such or introducing the components of said DNA constructs successively in the predetermined order into a vector DNA.

The construction of the plasmids according to the invention is performed applying conventional ligation techniques. The components of the plasmids are linked through common restriction sites and/or by means of synthetic linker molecules and/or by blunt end ligation.

A promoter of the invention may be used in transformed yeast, for example *Saccharomyces cerevisiae* or *Schizosaccharomyces pombe*, or in any other host in which the promoter is found to be effective. Fungal cells include the genera *Pichia*, *Saccharomyces*, *Kluyveromyces*, *Candida*, *Torulopsis*, *Hansenula*, *Schizosaccharomyces*, *Citero-*

myces, *Pachysolen*, *Debaromyces*, *Metschnikowia*, *Rhodospodium*, *Leucosporidium*, *Botryosascus*, *Sporidiobolus*, *Endomycopsis*, and the like. Preferred genera are those selected from the group consisting of *Pickia*, *Saccharomyces*, *Kluyveromyces*, *Yarrowia* and *Hansenula*, because the ability to manipulate the DNA of these yeasts has, at present, been more highly developed than for the other genera mentioned above. Examples of *Saccharomyces* are *Saccharomyces cerevisiae*, *Saccharomyces italicus* and *Saccharomyces rouxii*. Examples of *Kluyveromyces* are *Kluyveromyces fragilis* and *Kluyveromyces lactis*. Examples of *Hansenula* are *Hansenula polymorpha*, *Hansenula anomala* and *Hansenula capsulata*. *Yarrowia lipolytica* is an example of a suitable *Yarrowia* species. Filamentous fungi include *Aspergillus niger*.

Fungal cells can be transformed by: (a) digestion of the cell walls to produce spheroplasts; (b) mixing the spheroplasts with transforming DNA (derived from a variety of sources and containing both native and non-native DNA sequences); and (c) regenerating the transformed cells. The regenerated cells are then screened for the incorporation of the transforming DNA.

It has been demonstrated that fungal cells of the genera *Pichia*, *Saccharomyces*, *Kluyveromyces*, *Yarrowia* and *Hansenula* can be transformed by enzymatic digestion of the cells walls to give spheroplasts; the spheroplasts are then mixed with the transforming DNA and incubated in the presence of calcium ions and polyethylene glycol, then transformed spheroplasts are regenerated in regeneration medium.

Methods for the transformation of *S. cerevisiae* are taught generally in EP 251 744, EP 258 067 and WO 90/01063, all of which are incorporated herein by reference.

Alternatively, the transformation of yeast with the hybrid vectors may be accomplished according to the method described by Hinnen *et al* [*Proc. Natl. Acad. Sci. USA* 75, 1929 (1978)]. This method can be divided into three steps:

(1) Removal of the yeast cell wall or parts thereof using various preparations of glucosidases, such as snail gut juices (e.g. Glusulase[®] or Helicase[®]) or enzyme mixtures obtained from microorganisms (eg Zymolyase[®]) in osmotically stabilized solutions (eg 1M sorbitol).

(2) Treatment of the "naked" yeast cells (spheroplasts) with the DNA vector in the presence of PEG (polyethylene glycol) and Ca²⁺ ions.

(3) Regeneration of the cell wall and selection of the transformed cells in a solid layer of agar. This regeneration is conveniently done by embedding the spheroplasts into agar. For example, molten agar (about 50°C) is mixed with the spheroplasts. Upon cooling the solution to yeast growth temperatures (about 30°C), a solid layer is obtained. This agar layer is to prevent rapid diffusion and loss of essential macromolecules from the spheroplasts and thereby facilitates regeneration of the cell wall. However, cell wall regeneration may also be obtained (although at lower efficiency) by plating the spheroplasts onto the surface of preformed agar layers.

Preferably, the regeneration agar is prepared in a way to allow regeneration and selection of transformed cells at the same time. Since yeast genes coding for enzymes of amino acid biosynthetic pathways are generally used as selective markers (*-supra*), the regeneration is preferably performed in yeast minimal medium agar. If very high efficiencies of regeneration are required the following two step procedure is advantageous:

- (1) regeneration of the cell wall in a rich complex medium, and
- (2) selection of the transformed cells by replica plating the cell layer onto selective agar plates.

When the DNA vector is a linear DNA vector used for transforming eukaryotic host cells, transformation is preferably done in the presence of a second vector containing a selective marker for yeast. This cotransformation allows enrichment for those host cells which have taken up DNA that cannot be directly selected for. Since competent cells take up any type of DNA a high percentage of cells transformed with a selective vector will also harbour any additional DNA (such as the above linear DNA vector). The transformed host cells can be improved in production of the desired polypeptide by mutation and selection using methods known in the art. The mutation can be effected, for example, by U.V. irradiation or suitable chemical reagents. Strains which are deficient in protease A and B are particularly preferred; such strains are generally available.

The heterologous coding sequence may encode any desired polypeptide, including oligopeptides. The polypeptide may be fibronectin or a portion thereof (for example the collagen or fibrin-binding portions described in EP 207 751), urokinase, pro-urokinase, the 1-368 portion of CD4 (D Smith *et al* (1987) *Science* 238, 1704-1707) platelet derived growth factor (Collins *et al* (1985) *Nature* 316, 748-750), transforming growth factor β (Derynck *et al* (1985) *Nature* 316, 701-705), the 1-272 portion of Von Willebrand's Factor (Bonham *et al*, *Nucl. Acids Res.* 145 7125-7127), the Cathepsin D fragment of fibronectin (585-1578), α_1 -antitrypsin, plasminogen activator inhibitors, factor VIII, α -globin, β -globin, myoglobin, nerve growth factor, LACI (lipoprotein-associated coagulation inhibitor) (Broze, G. J. (1990) *Bio-*

chem. 29, 7539-7546), lactoferrin (Fletcher, J. in "Iron in Immunity, Cancer & Inflammation" 1989, Wiley & Sons, Eds. de Sousa, M. & Brock, J. H.) or platelet-derived endothelial cell growth factor (PDECGF) (Ishikawa, F. (1989) *Nature* 338, 557-562) or a conservative variant of any of these. The polypeptide may also be a fusion of HSA or an N-terminal portion thereof and any other polypeptide, such as those listed above. Preferably, the polypeptide is a naturally-occurring human serum albumin, a modified human serum albumin or a fragment of either, such modified forms and fragments being termed "variants", or is α - or β -globin. These variants include all forms or fragments of HSA which fulfill at least one of the physiological functions of HSA and which are sufficiently similar to HSA, in terms of structure (particularly tertiary structure) as to be regarded by the skilled man as forms or fragments of HSA.

In particular, variants or fragments of HSA which retain at least 50% of its ligand-binding properties (preferably 80%, or 95%), for example with respect to bilirubin or fatty acids, and/or at least 50% (preferably 80% or 90%) of its oncotic action are encompassed. Such properties are discussed in Brown, J R & Shockley, P (1982) in *Lipid-Protein Interactions* 1, 26-68, Ed. Jost, P C & Griffith, O H.

The portion of HSA disclosed in EP 322 094 is an example of a useful fragment of HSA which may be expressed by use of a promoter of the invention.

The polypeptide may initially be expressed as a fusion with a secretion leader sequence. In the case of HSA, this may, for example, be the natural HSA leader, the leader from the *S. cerevisiae* α mating factor, the *Kluyveromyces fragilis* killer toxin leader or a fusion between the natural HSA leader and either of the said yeast leaders. Thus, the leader may be either of SEQ4 and SEQ5 or conservatively modified variations of either sequence, as described in WO 90/01063.

The host cell may be fermented to express the desired polypeptide in known ways. The polypeptide may be purified by known techniques, for example (if the polypeptide is not secreted) separating off the cells, lysing them, collecting the supernatant, concentrating it and chromatographically separating the polypeptide.

The promoter of the invention is de-repressed by the absence of complex carbon sources (whether or not glycerol and ethanol are present), which is advantageous in large scale yeast culture. Thus, the invention provides a process for growing the transformed yeast to a high mass and then inducing expression of the desired polypeptide by allowing the medium to become exhausted of complex carbon sources and adding a simpler carbon source such as glycerol or ethanol.

Preferred aspects of the invention will now be described by way of example and with reference to the accompanying drawings, in which:

Figures 1 to 9 are respective restriction maps of plasmids pXL5, pAYE274, pAYE275, pAYE334, pAYE276, pAYE323, pAYE324, pSAC35 and pAYE321;

Figure 10 is a photograph of a gel showing labelled RNA from a cell culture at differing times;

Figure 11 is a graph showing the time course of expression of the glycerol-3-phosphate dehydrogenase promoter corresponding to Figure 10;

Figure 12 is a restriction map of plasmid pDXL200;

Figure 13 is a restriction map of plasmid pDVX2; and

Figure 14 is a restriction map of plasmid pDVX4.

Introduction

Strains and Culture Conditions

Escherichia coli DH5 α (F⁻, ϕ 80d Δ lacZdeltaM15, delta(lacZYA-argF) U169, *recA1*, *endA1*, *hsdR17* (*r_K*⁻, *m_K*⁺), *supE44*, lambda, *thi-1*, *gyrA*, *relA1*) was used for plasmid constructions. *E. coli* XL1-blue (Stratagene, *endA1*, *hsdR17* (*r_K*⁻, *m_K*⁺), *supE44*, *thi-1*, lambda, *recA1*, *gyrA96*, *relA1*.(lac-), [F', *proAB*, *lad*⁹ ZdeltaM15, Tn10, (tet^r)] was used for the propagation of M13 vectors. *Saccharomyces cerevisiae* DB1 *cir*⁺ (a, *leu2*) was used as the recombinant albumin expression host. Other *S. cerevisiae* strains used were AH22 *cir*⁺ (a, *can1*, *leu2*, *his4*); BJ1991 *cir*⁺ (α , *prb1-1122*, *pep4-3*, *leu2*, *trp1*, *ura3-52*) and S22 *cir*⁺ (a, *ade1*, *ade2*, *ura1*, *his7*, *tyr1*, *lys7*, *gal1*, *gut2*). Yeast cells were grown at 30°C on YEP (1% (w/v) yeast extract, 2% (w/v) bactopeptone) nutrient agar supplemented with the appropriate carbon source. *S. cerevisiae* transformants were grown in 10ml YEP, 2% (w/v) sucrose in 50ml conical shake flasks at 30°C, 200rpm for 72 hours. HSA antibody plates were prepared by cooling YEP containing 1% (w/v) electrophoresis grade agarose to 50°C. Rabbit anti-human albumin antiserum (Cambio, Cambridge, United Kingdom) was then added to 2.5% (v/v)

along with the appropriate carbon source and the nutrient medium poured into a Petri dish and allowed to cool.

DNA Manipulations

Standard DNA manipulation techniques were used (Maniatis *et al*, 1982: Molecular Cloning, A Laboratory Manual; Cold Spring Harbor; Sambrook *et al* 1989 (2nd edition). DNA fragments were routinely recovered from agarose gels by centrifugation (Vogelstein, B., *Anal. Biochem.* **160** (1987) 115-118). Radiolabelled DNA was prepared using [α - 32 P] dATP (Amersham International PLC) and the random primer labelling procedure (Feinburg, A P and Vogelstein, B., *Anal. Biochem.* **137**, (1984) 266-267). Restriction endonucleases, T4 DNA ligase, T4 DNA Polymerase and *E. coli* DNA polymerase I (Klenow fragment) were obtained from Boehringer-Mannheim.

The glycerol-3-phosphate dehydrogenase yeast promoter fragment was obtained from a genomic library of fragments obtained by *Bgl*II restriction of yeast DNA. The *Bgl*II restriction fragments are inserted into a unique *Bgl*II site of a plasmid containing the Herpes Simplex thymidine kinase (TK) gene. Only when promoter fragments are cloned in front of the thymidine kinase gene will yeast transformed with this plasmid grow in the presence of folate antagonists such as sulphanilamide and amethopterin, as described by Goodey *et al. Molecular and General Genetics* **204**, 505-511 (1986) which is incorporated herein by reference.

A plasmid having an active promoter was selected by measurement of thymidine kinase activity in the cell extract.

The promoter fragment was contained within a *Bgl*II restriction fragment of plasmid pXL5 (Figure 1). A 1.48kbp fragment of the glycerol-3-phosphate dehydrogenase promoter was sequenced (SEQ2). The promoter fragment was modified by the introduction of an *Sfi*I restriction endonuclease site on the 3' end of the yeast promoter:

NATURAL	GTACACCCCCCCCCTCCACAAACACAAATATTGATAATATAAAGATG	
ATG ENVIRONMENT		Met
		└───┘
		TRANSLATION
	** **	
MODIFIED	GTACGGCCCCCCCCGGCCACAAACACAAATATTGATAATATAAAGATG	
ATG ENVIRONMENT	┌──────────┘	Met
	<i>Sfi</i> I	└───┘
		TRANSLATION

These two sequences are SEQ6 and SEQ7 respectively.

EXAMPLE I: Expression of recombinant Human Serum Albumin (rHA)

A 282bp *Pst*I-*Rsa*I fragment of the promoter of the invention (ie from the CTGCAG at position 1031-1036 to the GTAC at 1314-1317 of SEQ1) and a 56bp double stranded oligonucleotide linker

5' -ACGGCCCCCCCCGGCCACAAACACAAATATTGATAATATAAAG	ATG	AAG	TGG	GTA
3' -TGCCGGGGGGGGCCGGTGTGTTGTGTTTATAACTATTATATTTTC	TAC	TTC	ACC	CAT
-5'				
TCGA-3'				

(the 5'-3' strand of which constitutes SEQ12) were inserted between the *Pst*I and *Hind*III site of M13mp18 (Yanisch-Perron *et al*, 1985, *Gene* **33**, 103-109) generating plasmid pAYE274 (Figure 2), so introducing a unique *Sfi*I 5' to the translation initiation site. Plasmid pAYE274 was linearised with *Eco*RI and *Pst*I and recircularised with the 2.3kb *Eco*RI-*Pst*I fragment from pXL5 (Figure 1) generating pAYE275 (Figure 3). This was digested with *Eco*RI-*Hind*III and the 2.3kb promoter fragment purified.

The construction of plasmid pAYE334, which is used in the next stages of the work, has been described in our co-pending UK patent application No 8927480.7 but is repeated here.

Plasmid pAAH5 (Goodey *et al.* 1987: In *Yeast Biotechnology*, 401-429, Edited by Berry, D.R., Russell, I. and Stewart, G.G. Published by Allen and Unwin) was linearised by partially digesting with *Bam*HI. The 5' protruding ends were blunt-ended with T4 DNA polymerase and ligated with the double-stranded oligonucleotide linker:

5' -GCGGCCGC-3'
3' -CGCCGGCG-5'

NotI

A recombinant plasmid pAYE334 (Figure 4) was selected in which a *NotI* restriction site had replaced the *BamHI* site at the 3' end of the *ADH1* terminator.

The modified promoter fragment from pAYE275 (Fig 3) and a 450bp *HindIII*-*NotI* *ADH1* terminator fragment from pAYE334 were ligated into pAT153 (Twigg and Sherratt, 1980) which itself had been modified by the introduction of *NotI* recognition site (5'-GCGGCCGC-3') into and so destroying the *BamHI* site, generating pAYE276 (Figure 5).

Plasmid pAYE276 was linearised with *EcoRI*-*SstII*, the 3' recessed ends filled in the T4 DNA Polymerase and dNTP and recircularised with excess *NotI* linker (5'-GCGGCCGC-3') generating plasmid pAYE323 (Figure 6). This plasmid was linearised with *HindIII* and recircularised with a double stranded oligonucleotide linker:

5' -AGCTTTATTTCCCTTCTTTTCTCTTTAGCTCGGCTTATTCCAGGAGCTTGGATAA
3' -AATAAAGGGAAGAAAAAGAGAAATCGAGCCGAATAAGGTCTCGAACCTATT

HindIII

AAGA-3'

TTCT-5'

(the 5'-3' strand of which constitutes SEQ13) and a 1.9kbp HA cDNA fragment liberated from *XhoI* linearised mp19.7 (EP-A-201 239), blunt ended with S1 nuclease and then digested with *HindIII*, to create plasmid pAYE324 (Figure 7).

The 3.72kbp *NotI* restriction fragment created in plasmid pAYE324 (Figure 7) may then be transferred into a suitable yeast replicating vector that contains a unique *NotI* restriction site (for example pSAC35, Figure 8), to create a plasmid such as pAYE321 (Figure 9).

Plasmid pSAC35 is a derivative of pSAC3 described by Chinery and Hinchliffe (1989) *Curr. Genet.* **16**, 21-25, and in EP 286424. The *LEU2* selectable marker is a 1.95 kbp *SalI* - *HpaI* fragment from YEP13, (Broach J R, *et al* (1979) *Cell* **16**, 827-839) inserted into the *SnaBI* site of pSAC3. The *LEU2* gene possesses a unique *Tth111I* site. Following digestion with this enzyme the 5' protruding ends were removed by treatment with the Klenow fragment of *E. coli* DNA Polymerase I. The insertion of a *NotI* recognition site to generate pSAC35 was achieved by ligating the blunt end linearised DNA with a double stranded oligonucleotide of the sequence,

5' -GCGGCCGC-3'
3' -CGCCGGCG-5'

Those skilled in the art will recognise a large number of techniques for modifying DNA segments which code for a wide variety of proteins for insertion into an *SfiI* restriction site.

This Example describes an HSA secretion vector (pAYE321) incorporating a promoter of the invention. This vector has been used to transform five different yeast strains: all five strains secreted HSA into the culture supernatant. The timing of HSA expression under the control of the promoter has also been studied. HSA mRNA is first detected when the cells have reached late logarithmic growth. High levels of HSA mRNA are maintained even when the culture has entered stationary phase.

Plasmid pAYE324 (Figure 7) is a pAT153-based vector which possesses the entire promoter/HSA secretion cassette flanked by *NotI* restriction sites. The 3.715kbp secretion cassette contains the following features:

i) A 1.35kbp promoter fragment which includes the native promoter ATG environment except that four nucleotide substitutions have been incorporated at a site between 30 and 40bp upstream of the ATG as described above (SEQ7). These substitutions introduce a unique *SfiI* restriction site in the 3' region of the promoter.

ii) The natural HSA/ α -factor fusion leader sequence (WO 90/01063) directing the secretion of mature HSA.

iii) The yeast alcohol dehydrogenase *ADH1* terminator region.

The 3.715kbp *NotI* promoter/HSA secretion cassette was purified and inserted into the unique *NotI* cloning site of pSAC35 (Figure 8) to generate plasmid pAYE321 (Figure 9).

Five [*cir*⁺] strains were transformed to leucine prototrophy with plasmid pAYE321, namely Strain 1 [*cir*⁺], Strain 2 [*cir*⁺], Strain 3 [*cir*⁺], Strain 4 [*cir*⁺] and Strain 5 [*cir*⁺]. Transformation was performed essentially as described by Beggs (Nature, **275** (1978) 104-109) except for the following modifications. Transforming DNA in 10µl deionised H₂O was gently mixed with 50µl of spheroplasts in 1.2M sorbitol, 10mM CaCl₂ and 12.5µl 20% (w/v) PEG 3350 (Sigma), 10mM CaCl₂, 10mM Tris/HCl (pH7.5) and held on ice for 15 minutes. After adding a further 500µl of 20% (w/v) PEG 3350, 10mM CaCl₂, 10mM Tris/HCl (pH7.5) the spheroplasts were gently mixed with 5ml of 1.2M sorbitol selective agar medium and plated out. Two independent transformants from each strain were grown for 72 hours, 200 rpm shaking, at 30°C in 10ml of YEP (1% w/v yeast extract, 2% w/v bacto-peptone and 2% w/v glucose).

HSA was detected in the culture supernatants of all the transformants, showing that the promoter can direct the expression/secretion of heterologous proteins in yeast.

EXAMPLE 2: Timing of Expression

A one-litre shake flask containing 400ml of YEP, 2% (w/v) glucose was inoculated with Strain 1 pAYE321 and incubated at 30°C, 200 rpm. Samples (20ml) were removed at 24 hours, 48 hours, 72 hours, 96 hours and 120 hours post inoculation. At each time point, the optical density of the culture and secreted HSA were determined. The sample was then separated by centrifugation into a cell pellet and culture supernatant. The level of HSA secreted into the supernatant was measured by rocket gel electrophoresis and RNA extracted from the cell pellet. The RNA from each time point was separated into its individual components by gel electrophoresis, Northern blotted and probed with radio-labelled DNA homologous to the PGK and HSA structural genes. RNA was extracted from yeast cells as described by Linquist (Nature **293** (1981) 311-314). 10µg of total yeast RNA was resolved on a 1.0% agarose-formaldehyde gel and vacuum blotted from 20 x SSPE onto a Pall bio-dyne nylon membrane, and UV cross-linked according to KroczeK and Siebet (Anal. Biochem. **184** (1990) 90-95). Hybridisation was performed at 6 x SSPE, 5 x Denhardt's, 0.1% (w/v) SDS, 100µg/ml denatured herring sperm DNA, at 50°C for 18 hours. Washing stringency was 0.2 x SSPE, 0.1% (w/v) SDS, 50°C.

The results are illustrated in Figure 10. Figure 11 shows the optical density and level of rHA during the experiment. At the first timepoint 24 hrs post inoculation, PGK mRNA is observed; however, neither secreted HSA nor HSA mRNA are detected. At the second time point, 48 hrs post inoculation, both PGK and HSA mRNA are detected within the cell. The HSA mRNA is available for translation because secreted HSA is observed in the culture supernatant. At the next three time points, 72 hrs, 96 hrs and 120 hrs post inoculation, only HSA mRNA is observed and the PGK mRNA has disappeared. The level of HSA observed in the culture supernatant has increased from the previous time point, but no further increase is observed. The following conclusions can be drawn.

i) The HSA gene is not expressed during the early growth phase and does not mirror PGK expression.

ii) The HSA gene is expressed and HSA is secreted during the late logarithmic and stationary growth phase.

iii) HSA mRNA levels are maintained during stationary phase.

Furthermore, the timing of expression can be manipulated in the controlled environment of a fermentation vessel, be it batch, fed-batch or continuous culture. When repressing carbon sources such as sucrose or glucose are supplied as the sole carbon source, the expression of the heterologous protein is repressed. Consequently the growth of the host organism is not impaired by the synthesis of the heterologous protein. At a point predetermined by the operator the sucrose or glucose is replaced by a non-repressing carbon source such as glycerol or ethanol. Under these conditions the expression of the heterologous protein is de-repressed. Consequently production can be regulated in such a way as to optimise the synthesis of the desired product.

EXAMPLE 3: Expression with various carbon sources

Strain 1 pAYE321 was grown for 72 hrs in 10 ml YEP, 200 rpm, 30°C supplemented with various carbon sources. In the control experiment sucrose is supplied instead of glucose but the final HSA secretion levels are identical. In all the other experiments a stimulation of HSA secretion is observed. The results are given in Table 1 below. The best carbon source would appear to be a combination of 1% (v/v) ethanol and 1% (v/v) glycerol. Although the stimulating effect at first sight does not appear very great it must be remembered that the value achieved on sucrose as a carbon

Carbon Source			HSA secreted into culture supernatant (arbitrary units)
% sucrose (w/v)	% glycerol (v/v)	% ethanol (v/v)	
2	-	-	7.5
-	2	-	9.0
-	-	2	9.5
-	1.5	0.5	10.5
-	1.0	1.0	12.5
-	0.5	1.5	11.0

This example describes plasmid pDVX4 designed for expressing and secreting the *S. cerevisiae* var *diastaticus* glucoamylase.

The initial step involved the construction of a generalised brewing yeast vector pDXL200 (Fig 12) which contained the following DNA sequences:

- b) A synthetic oligonucleotide linker containing restriction enzyme sites for *SfiI*, *BglII* and *HindIII*:

*Sfi*II
*Bgl*II
*Hind*III

5' G T A C G G C C C C C C C G G C C A G A T C T A A G C T T C

Derived Sequence

3' C A T G C C G G G G G G C C G G T C T A G A T T C G A A

Oligos 5' C G G C C A G A T C T A 3' (12)
Synthesized: 3' G G G G C C G G T C T A G A T T C G A 5' (12)

The 5'-3' modified region and the two oligonucleotides are listed as SEQ14, SEQ8 and SEQ9 respectively.

- 9

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Eds. Strathern, J N, E W Jones and J R Broach). Cold Spring Harbor, p445.) The full DNA sequence of 2 μ m DNA is also known (Hartley, J L and Donelson, J E (1980). Nature. **226**, 860).

The *DEX1* gene which codes for glucoamylase was isolated from *S. cerevisiae* var *diastaticus* (Meaden P K *et al*, (1985) *Gene*. **34**, 325 and PCT/GB85/00599; Pardo *et al* (1988 FEBS. Lett. **239**, 179-184 describe the *DEX1* promoter and part of the open reading frame). A 2.75kbp *Bgl*II fragment carrying the *DEX1* gene was cloned into the unique *Bgl*II site in pDXL200 and the DNA sequence is represented as SEQ10, with the protein encoded thereby appearing as SEQ11. The resulting plasmid, pDVX2 (Fig. 13), was digested with *Xba*I to remove the smaller fragment (2.7kbp) containing the bacterial DNA. After gel purification, the larger *Xba*I fragment was transformed into brewing yeast and this plasmid designated pDVX4 (Fig. 14).

Expression of *DEX1*

Brewing yeast strains transformed to copper resistance with plasmid pDVX4 were assayed for glucoamylase production by measuring glucose released from starch using the hexokinase-UV assay (Boehringer-Mannheim). In all cases copper resistant transformants produced significant quantities of extracellular glucoamylase.

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS :

- (A) LENGTH: 1357 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: N

(iv) ANTI-SENSE: N

(vi) ORIGINAL SOURCE:

(A) ORGANISM: *Saccharomyces cerevisiae*

(ix) FEATURE:

(A) NAME/KEY: promoter

(B) LOCATION: 42..52

(D) OTHER INFORMATION: /function= "RNA POLIII promoter box A"

(ix) FEATURE:

(A) NAME/KEY: promoter

(B) LOCATION: 86..96

(D) OTHER INFORMATION: /function= "RNA POLIII Promoter box B"

(ix) FEATURE:

(A) NAME/KEY: promoter

(B) LOCATION: 113..118

(D) OTHER INFORMATION: /function= "RNA POLIII Terminator"

(ix) FEATURE:

(A) NAME/KEY: protein_bind

(B) LOCATION: 1091..1103

(D) OTHER INFORMATION: /bound_moiety= "RAPI/GRFI/TUFI"

(ix) FEATURE:

(A) NAME/KEY: protein_bind

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(B) LOCATION: 1106..1118
(D) OTHER INFORMATION: /bound_moiety= "RAPI/GRFI/TUFI"

(ix) FEATURE:

(A) NAME/KEY: misc_signal
(B) LOCATION: 1176..1241
(D) OTHER INFORMATION: /function= "Pyrimidine (CT) block"

(ix) FEATURE:

(A) NAME/KEY: TATA_signal
(B) LOCATION: 1326..1335
(D) OTHER INFORMATION:

(ix) FEATURE:

(A) NAME/KEY: misc_signal
(B) LOCATION: 1369..1406
(D) OTHER INFORMATION: /function= "Pyrimidine (CT) block"

(ix) FEATURE:

(A) NAME/KEY: misc_signal
(B) LOCATION: 1418..1421
(D) OTHER INFORMATION: /function= "CRAG box"

(ix) FEATURE:

(A) NAME/KEY: misc_signal
(B) LOCATION: 1425..1429
(D) OTHER INFORMATION: /function= "CCAAT box"

(ix) FEATURE:

(A) NAME/KEY: misc_feature
(B) LOCATION: 1031..1036
(D) OTHER INFORMATION: /function= "PstI restriction site"

(ix) FEATURE:

(A) NAME/KEY: misc_feature
(B) LOCATION: 1314..1317
(D) OTHER INFORMATION:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

CCGCGGTGCC	GAGATGCAGA	CGTGGCCAAC	TGTGTCTGCC	GTCGCAAAAT	GATTTGAATT	60
TTGCGTCGCG	CACGTTTCTC	ACGTACATAA	TAAGTATTTT	CATACAGTTC	TAGCAAGACG	120
AGGTGGTCAA	AATAGAAGCG	TCCTATGTTT	TACAGTACAA	GACAGTCCAT	ACTGAAATGA	180
CAACGTACTT	GACTTTTCAG	TATTTTCTTT	TTCTCACAGT	CTGGTTATTT	TTGAAAGCGC	240

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ACGAAATATA TGTAGGCAAG CATTTTCTGA GTCTGCTGAC CTCTAAAATT AATGCTATTG 300
TGCACCTTAG TAACCCAAGG CAGGACAGTT ACCTTGCGTG GTGTACTAT GGCCGGAAGC 360
5 CCGAAAGAGT TATCGTTACT CCGATTATTT TGTACAGCTG ATGGGACCTT GCCGTCTTCA 420
TTTTTTTTTT TTTTCACCTA TAGAGCCGGG CAGAGCTGCC CGGCTTAACT AAGGGCCGGA 480
AAAAAACGG AAAAAAGAAA GCCAAGCGTG TAGACGTAGT ATAACAGTAT ATCTGACACG 540
10 CACGTGATGA CCACGTAATC GCATCGCCCC TCACCTCTCA CCTCTCACC GCTGACTCAGC 600
TTCATAAAA AGGAAAATAT ATACTCTTTT CCAGGCAAGG TGACAGCGGT CCCCGTCTCC 660
TCCACAAAGG CCTCTCCTGG GGTGTGAGCA AGTCTAAGTT TACGTAGCAT AAAAATTCTC 720
15 GGATTGCGTC AAATAATAAA AAAAGTAACC CCACTTCTAC TTCTACATCG GAAAAACATT 780
C .TTCACAT ATCGTCTTTG GCCTATCTTG TTTTGTCTC GGTAGATCAG GTCAGTACAA 840
ACGCAACACG AAAGAACAAA AAAAGAAGAA AACAGAAGGC CAAGACAGGG TCAATGAGAC 900
20 TGTGTGCTCT CTA CTGTGCTCC TATGTCTCTG GCCGATCAG CGCCATTGTC CCTCAGAAAC 960
AAATCAAACA CCCACACCCC GGGCACCCAA AGTCCCCACC CACACCACCA ATACGTAAAC 1020
GGGGCGCCCC CTGCAGGCCC TCCTGCGCGC GGCCTCCCGC CTTGCTTCTC TCCCCTTCCT 1080
25 TTTCTTTTTC CAGTTTTTCC TATTTTGTCC CTTTTTCCGC ACAACAAGTA TCAGAATGGG 1140
TTCATCAAAT CTATCCAACC TAATTCGCAC GTAGACTGGC TTGGTATTGG CAGTTTCGTA 1200
GTTATATATA TACTACCATG AGTGAAACTG TTACGTTACC TTAAATTCTT TCTCCCTTTA 1260
30 ATTTTCTTTT ATCTTACTCT CCTACATAAG ACATCAAGAA ACAATTGTAT ATTGTACACC 1320
CCCCCCTCC ACAAAACAAA ATATTGATAA TATAAAG 1357

35 (2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

40 (A) LENGTH: 1483 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

45 (ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:

50 (A) NAME/KEY: misc_feature
(B) LOCATION: 123..124
(D) OTHER INFORMATION: /function= "SstII restriction site"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

AAGAAAGATT CTCGGTAACG ACCATACAAA TATTGGGCGT GTGGCGTAGT CGGTAGCGCG 60
55 CTCCCTTAGC ATGGGAGAGG TCTCCGGTTC GATTCCGGAC TCGTCCAAAT TATTTTTTAC 120

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	TTTCCGCGGT GCGGAGATGC AGACGTGGCC AACTGTGTCT GCCGTCGCAA AATGATTTGA	180
	ATTTTGCGTC GCGCACGTTT CTCACGTACA TAATAAGTAT TTTCATACAG TTCTAGCAAG	240
5	ACGAGGTGGT CAAAATAGAA GCGTCCTATG TTTTACAGTA CAAGACAGTC CATACTGAAA	300
	TGACAACGTA CTTGACTTTT CAGTATTTTC TTTTCTCAC AGTCTGGTTA TTTTGAAG	360
	CGCACGAAAT ATATGTAGGC AAGCATTTTC TGAGTCTGCT GACCTCTAAA ATTAATGCTA	420
10	TTGTGCACCT TAGTAACCCA AGGCAGGACA GTTACCTTGC GTGGTGTTAC TATGGCCGGA	480
	AGCCCGAAAG AGTTATCGTT ACTCCGATTA TTTTGTACAG CTGATGGGAC CTTGCCGTCT	540
	TCATTTTTTT TTTTTTTCAC CTATAGAGCC GGGCAGAGCT GCCCGGCTTA ACTAAGGGCC	600
15	GGAAAAAA CCGAAAAAG AAAGCCAAGC GTGTAGACGT AGTATAACAG TATATCTGAC	660
	ACACGCTGA TGACCACGTA ATCGCATCGC CCCTCACCTC TCACCTCTCA CCGCTGACTC	720
	AGCTTCACTA AAAAGGAAAA TATATACTCT TTCCAGGCA AGGTGACAGC GGTCCCCGTC	780
20	TCCTCCACAA AGGCCTCTCC TGGGGTTTGA GCAAGTCTAA GTTTACGTAG CATAAAAATT	840
	CTCGGATTGC GTCAAATAAT AAAAAAGTA ACCCCACTTC TACTTCTACA TCGGAAAAAC	900
25	ATTCCATTCA CATATCGTCT TTGGCCTATC TTGTTTGTG CTCGGTAGAT CAGGTCAGTA	960
	CAAACGCAAC ACGAAAGAAC AAAAAAGAA GAAACAGAA GGCCAAGACA GGGTCAATGA	1020
	GACTGTTGTC CTCCTACTGT CCCTATGTCT CTGGCCGATC ACGCGCCATT GTCCCTCAGA	1080
30	AACAAATCAA ACACCCACAC CCCGGGCACC CAAAGTCCCC ACCCACACCA CCAATACGTA	1140
	AACGGGGCGC CCCCTGCAGG CCCTCCTGCG CGCGGCCTCC CGCCTTGCTT CTCTCCCCTT	1200
	CCCTTTCTTT TTCCAGTTT CCCTATTTTG TCCCTTTTTC CGCACAACAA GTATCAGAAT	1260
35	GGGTTTCATCA AATCTATCCA ACCTAATTCT CACGTAGACT GGCTTGGTAT TGGCAGTTTC	1320
	GTAGTTATAT ATATACTACC ATGAGTGAAA CTGTTACGTT ACCTTAAATT CTTTCTCCCT	1380
	TTAATTTTCT TTTATCTTAC TCTCCTACAT AAGACATCAA GAAACAATTG TATATTGTAC	1440
40	ACCCCCCCCC TCCACAAACA CAAATATTGA TAATATAAAG ATG	1483

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 380 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: N

(iv) ANTI-SENSE: N

(ix) FEATURE:

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(A) NAME/KEY: misc_feature
(B) LOCATION: 54..59
(D) OTHER INFORMATION: /function= "PstI restriction site"

(ix) FEATURE:

(A) NAME/KEY: misc_feature
(B) LOCATION: 54..59
(D) OTHER INFORMATION: /function= "PstI restriction site"

(ix) FEATURE:

(A) NAME/KEY: misc_feature
(B) LOCATION: 337..340
(D) OTHER INFORMATION: /function= "RsaI restriction site"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

CCCCGGGCACC CAAAGTCCCC ACCCACACCA CCAATACGTA AACGGGGCGC CCCCTGCAGG	60
CCTCTGCG CGCGGCCTCC CGCCTTGCTT CTCTCCCTT CCTTTTCTTT TTCCAGTTTT	120
CCCTATTTTG TCCCTTTTTC CGCACAAACA GTATCAGAAT GGGTTCATCA AATCTATCCA	180
ACCTAATTCG CACGTAGACT GGCTTGGTAT TGGCAGTTTC GTAGTTATAT ATATACTACC	240
ATGAGTGAAA CTGTTACGTT ACCTTAAATT CTTTCTCCCT TTAATTTTCT TTTATCTTAC	300
TCTCCTACAT AAGACATCAA GAAACAATTG TATATTGTAC ACCCCCCCCC TCCACAAACA	360
CAAATATTGA TAATATAAAG	380

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 24 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: N

(iv) ANTI-SENSE: N

(v) FRAGMENT TYPE: N-terminal

(ix) FEATURE:

(A) NAME/KEY: Peptide
(B) LOCATION: 1..24
(D) OTHER INFORMATION: /label= leader
/note= "Synthetic secretion leader sequence"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

Met Lys Trp Val Ser Phe Ile Ser Leu Leu Phe Leu Phe Ser Ser Ala
1 5 10 15

5 Tyr Ser Arg Ser Leu Asp Lys Arg
20

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 21 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: N

(iv) ANTI-SENSE: N

(v) FRAGMENT TYPE: N-terminal

(ix) FEATURE:

- (A) NAME/KEY: Peptide
- (B) LOCATION: 1..21
- (D) OTHER INFORMATION: /label= leader
/note= "Synthetic secretion leader sequence"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

35 Met Asn Ile Phe Tyr Ile Phe Leu Phe Leu Leu Ser Phe Val Gln Gly
1 5 10 15

Ser Leu Asp Lys Arg
20

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 47 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: N

(iv) ANTI-SENSE: N

(vi) ORIGINAL SOURCE:

- (A) ORGANISM: Saccharomyces cerevisiae

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(ix) FEATURE:

(A) NAME/KEY: exon

(B) LOCATION: 1..47

(D) OTHER INFORMATION: /note= "Natural ATG environment of the promoter"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

GTACACCCCC CCCCTCCACA AACACAAATA TTGATAATAT AAAGATG

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 47 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: N

(iv) ANTI-SENSE: N

(ix) FEATURE:

(A) NAME/KEY: modified_base

(B) LOCATION: 5

(D) OTHER INFORMATION:

(ix) FEATURE:

(A) NAME/KEY: modified_base

(B) LOCATION: 6

(D) OTHER INFORMATION:

(ix) FEATURE:

(A) NAME/KEY: modified_base

(B) LOCATION: 14

(D) OTHER INFORMATION:

(ix) FEATURE:

(A) NAME/KEY: modified_base

(B) LOCATION: 15

(D) OTHER INFORMATION:

(ix) FEATURE:

(A) NAME/KEY: -

(B) LOCATION: 5..17

(D) OTHER INFORMATION: /label= SfiI
/note= "SfiI restriction site"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

G: JGGCCCC CCCGGCCACA AACACAAATA TTGATAATAT AAAGATG

47

(2) INFORMATION FOR SEQ ID NO:8:

5

(i) SEQUENCE CHARACTERISTICS:

10

- (A) LENGTH: 12 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

15

(iii) HYPOTHETICAL: Y

(iv) ANTI-SENSE: N

(ix) FEATURE:

20

- (A) NAME/KEY: misc_feature
- (B) LOCATION: 1..12
- (D) OTHER INFORMATION: /function= "synthetic oligo used to create SEQ14"

25

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

CGGCCAGATC TA

12

(2) INFORMATION FOR SEQ ID NO:9:

30

(i) SEQUENCE CHARACTERISTICS:

35

- (A) LENGTH: 19 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

40

(ix) FEATURE:

45

- (A) NAME/KEY: misc_feature
- (B) LOCATION: 1..19
- (D) OTHER INFORMATION: /function= "synthetic oligo used to create SEQ14"
/note= "This oligo is complementary to SEQ8"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

AGCTTAGATC TGGCCGGGG

19

50

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:

55

- (A) LENGTH: 2754 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: linear

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(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: N

5 (iv) ANTI-SENSE: N

(vi) ORIGINAL SOURCE:

10 (A) ORGANISM: *Saccharomyces cerevisiae*
(B) STRAIN: *S. cerevisiae* var. *diastaticus* 5106-9A

(ix) FEATURE:

15 (A) NAME/KEY: misc_feature
(B) LOCATION: 98..103
(D) OTHER INFORMATION: /function= "StuI/BglII site"

(ix) FEATURE:

20 (A) NAME/KEY: CDS
(B) LOCATION: 126..2543
(D) OTHER INFORMATION:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

25 GATCTTTTGC TTCCTAAACT AAACCTATAA AAAGCACCCCT ATTCATCAGT TATAATCTCT 60
TGTCATGTTG TGGTTCTAAT TGAAAATATA CTATGGTAGG CCTCAAAAAT CCATATACGC 120

30

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	ACACT ATG CAA AGA CCA TTT CTA CTC GCT TAT TTG GTC CTT TCG CTT	167
	Met Gln Arg Pro Phe Leu Leu Ala Tyr Leu Val Leu Ser Leu	
	1 5 10	
5	CTA TTT AAC TCA GCT TTG GGT TTT CCA ACT GCA CTA GTT CCT AGA GGA	215
	Leu Phe Asn Ser Ala Leu Gly Phe Pro Thr Ala Leu Val Pro Arg Gly	
	15 20 25 30	
10	TCC TCC TCT AGC AAC ATC ACT TCG TCC GGT CCA TCT TCA ACT CCA TTC	263
	Ser Ser Ser Ser Asn Ile Thr Ser Ser Gly Pro Ser Ser Thr Pro Phe	
	35 40 45	
15	AGC TCT GCT ACT GAA AGC TTT TCT ACT GGC ACT ACT GTC ACT CCA TCA	311
	Ser Ser Ala Thr Glu Ser Phe Ser Thr Gly Thr Thr Val Thr Pro Ser	
	50 55 60	
20	TCA TCC AAA TAC CCT GGC AGT AAA ACA GAA ACT TCT GTT TCT TCT ACA	359
	Ser Ser Lys Tyr Pro Gly Ser Lys Thr Glu Thr Ser Val Ser Ser Thr	
	65 70 75	
25	ACC GAA ACT ACC ATT GTT CCA ACT ACA ACT ACG ACT TCT GTC ATA ACA	407
	Thr Glu Thr Thr Ile Val Pro Thr Thr Thr Thr Thr Ser Val Ile Thr	
	80 85 90	
30	CCA TCA ACA ACC ACT ATT ACC ACT ACG GTT TGC TCT ACA GGA ACA AAC	455
	Pro Ser Thr Thr Thr Ile Thr Thr Thr Val Cys Ser Thr Gly Thr Asn	
	95 100 105 110	
35	TCT GCC GGT GAA ACT ACT TCT GGA TGC TCT CCA AAG ACC ATT ACA ACT	503
	Ser Ala Gly Glu Thr Thr Ser Gly Cys Ser Pro Lys Thr Ile Thr Thr	
	115 120 125	
40	ACT GTT CCA TGT TCA ACC AGT CCA AGC GAA ACC GCA TCG GAA TCA ACA	551
	Thr Val Pro Cys Ser Thr Ser Pro Ser Glu Thr Ala Ser Glu Ser Thr	
	130 135 140	
45	ACC ACT TCA CCT ACC ACA CCT GTA ACT ACA GTT GTC GCA ACC ACC GTC	599
	Thr Thr Ser Pro Thr Thr Pro Val Thr Thr Val Val Ala Thr Thr Val	
	145 150 155	
50	GTT ACT ACT GAG TAT TCT ACT AGT ACA AAA CAA GGT GGT GAA ATT ACA	647
	Val Thr Thr Glu Tyr Ser Thr Ser Thr Lys Gln Gly Gly Glu Ile Thr	
	160 165 170	
55	ACT ACA TTT GTC ACC AAA AAC AGT CCA ACC ACT TAC CTA ACT ACA ATT	695
	Thr Thr Phe Val Thr Lys Asn Ser Pro Thr Thr Tyr Leu Thr Thr Ile	
	175 180 185 190	
60	GCT CCA ACT TCA TCA GTC ACT ACG GTT ACC AAT TTC ACC CCA ACC ACT	743
	Ala Pro Thr Ser Ser Val Thr Thr Val Thr Asn Phe Thr Pro Thr Thr	
	195 200 205	
65	ATT ACT ACT ACG GTT TGC TCT ACA GGA ACA AAC TCT GCC GGT GAA ACT	791
	Ile Thr Thr Thr Val Cys Ser Thr Gly Thr Asn Ser Ala Gly Glu Thr	
	210 215 220	
70	ACC TCT GGA TGC TCT CCA AAG ACT GTC ACA ACA ACT GTT CTT TGT TCA	839
	Thr Ser Gly Cys Ser Pro Lys Thr Val Thr Thr Thr Val Leu Cys Ser	
	225 230 235	

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	ACT	GGT	ACT	GGC	GAA	TAC	ACT	ACT	GAA	GCT	ACC	GCC	CCT	GTT	ACA	ACA	887
	Thr	Gly	Thr	Gly	Glu	Tyr	Thr	Thr	Glu	Ala	Thr	Ala	Pro	Val	Thr	Thr	
		240					245					250					
5	GCT	GTC	ACA	ACC	ACC	GTT	GTT	ACC	ACT	GAA	TCC	TCT	ACG	GGT	ACT	AAC	935
	Ala	Val	Thr	Thr	Thr	Val	Val	Thr	Thr	Glu	Ser	Ser	Thr	Gly	Thr	Asn	
	255					260				265						270	
10	TCC	GTC	GGT	AAG	ACG	ACA	ACT	AGT	TAC	ACA	ACA	AAG	TCT	GTA	CCA	ACC	983
	Ser	Val	Gly	Lys	Thr	Thr	Thr	Ser	Tyr	Thr	Thr	Lys	Ser	Val	Pro	Thr	
				275					280						285		
	ACC	TAT	GTA	TTT	GAC	TTT	GGC	AAG	GGC	ATT	CTC	GAT	CAA	AGC	TGC	GGC	1031
	Thr	Tyr	Val	Phe	Asp	Phe	Gly	Lys	Gly	Ile	Leu	Asp	Gln	Ser	Cys	Gly	
				290					295					300			
15	GGT	GTA	TTT	TCA	AAC	AAC	GGC	TCT	TCG	CAA	GTG	CAG	CTG	CGG	GAT	GTA	1079
	Gly	Val	Phe	Ser	Asn	Asn	Gly	Ser	Ser	Gln	Val	Gln	Leu	Arg	Asp	Val	
			305				310						315				
20	GTC	TTG	ATG	AAT	GGG	ACA	GTG	GTA	TAC	GAT	TCA	AAC	GGC	GCT	TGG	GAC	1127
	Val	Leu	Met	Asn	Gly	Thr	Val	Val	Tyr	Asp	Ser	Asn	Gly	Ala	Trp	Asp	
		320					325					330					
	AGT	AGT	GCG	CTG	GAG	GAG	TGG	CTC	CAG	CGA	CAG	AAA	AAA	GTT	TCC	ATC	1175
	Ser	Ser	Ala	Leu	Glu	Glu	Trp	Leu	Gln	Arg	Gln	Lys	Lys	Val	Ser	Ile	
	335					340				345						350	
25	GAA	AGA	ATA	TTT	GAA	AAT	ATT	GGG	CCC	AGC	GCC	GTG	TAT	CCG	TCT	ATT	1223
	Glu	Arg	Ile	Phe	Glu	Asn	Ile	Gly	Pro	Ser	Ala	Val	Tyr	Pro	Ser	Ile	
					355				360						365		
30	TTG	CCT	GGG	GTC	GTG	ATT	GCG	TCA	CCA	TCG	CAA	ACG	CAT	CCA	GAC	TAC	1271
	Leu	Pro	Gly	Val	Val	Ile	Ala	Ser	Pro	Ser	Gln	Thr	His	Pro	Asp	Tyr	
				370					375					380			
	TTG	TAC	CAA	TGG	ATA	AGG	GAC	AGC	GCG	TTG	ACG	ATA	AAC	AGT	ATT	GTC	1319
	Phe	Tyr	Gln	Trp	Ile	Arg	Asp	Ser	Ala	Leu	Thr	Ile	Asn	Ser	Ile	Val	
			385				390						395				
35	TCT	CAT	TCT	GCG	GAC	CCG	GCA	ATA	GAG	ACG	TTA	TTG	CAG	TAC	CTG	AAC	1367
	Ser	His	Ser	Ala	Asp	Pro	Ala	Ile	Glu	Thr	Leu	Leu	Gln	Tyr	Leu	Asn	
		400				405						410					
40	GTT	TCA	TTC	CAC	TTG	CAA	AGA	ACC	AAC	AAC	ACA	TTG	GGC	GCT	GGC	ATT	1415
	Val	Ser	Phe	His	Leu	Gln	Arg	Thr	Asn	Asn	Thr	Leu	Gly	Ala	Gly	Ile	
	415					420					425					430	
	GGT	TAC	ACT	AAC	GAT	ACA	GTG	GCT	TTG	GGA	GAC	CCT	AAG	TGG	AAC	GTC	1463
	Gly	Tyr	Thr	Asn	Asp	Thr	Val	Ala	Leu	Gly	Asp	Pro	Lys	Trp	Asn	Val	
					435					440					445		
45	GAC	AAC	ACG	GCT	TTC	ACG	GAA	CCT	TGG	GGT	CGT	CCT	CAA	AAC	GAT	GGC	1511
	Asp	Asn	Thr	Ala	Phe	Thr	Glu	Pro	Trp	Gly	Arg	Pro	Gln	Asn	Asp	Gly	
				450					455					460			
50	CCT	GCT	CTT	CGA	AGC	ATT	GCC	ATC	TTA	AAA	ATC	ATC	GAC	TAC	ATC	AAG	1559
	Pro	Ala	Leu	Arg	Ser	Ile	Ala	Ile	Leu	Lys	Ile	Ile	Asp	Tyr	Ile	Lys	
			465				470						475				

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	CAA	TCT	GGC	ACT	GAT	CTG	GGG	GCC	AAG	TAC	CCA	TTC	CAG	TCC	ACC	GCA	1607
	Gln	Ser	Gly	Thr	Asp	Leu	Gly	Ala	Lys	Tyr	Pro	Phe	Gln	Ser	Thr	Ala	
	480						485					490					
5	GAT	ATC	TTT	GAT	GAT	ATT	GTA	CGT	TGG	TAC	CTG	AGG	TTC	ATT	ATT	GAC	1655
	Asp	Ile	Phe	Asp	Asp	Ile	Val	Arg	Trp	Tyr	Leu	Arg	Phe	Ile	Ile	Asp	
	495					500					505					510	
10	CAC	TGG	AAT	TCT	TCC	GGA	TTT	GAT	CTA	TGG	GAG	GAA	GTC	AAT	GGC	ATG	1703
	His	Trp	Asn	Ser	Ser	Gly	Phe	Asp	Leu	Trp	Glu	Glu	Val	Asn	Gly	Met	
					515					520					525		
	CAT	TTC	TTT	ACT	TTA	CTG	GTA	CAA	CTG	TCT	GCA	GTG	GAC	AGG	ACG	CTG	1751
	His	Phe	Phe	Thr	Leu	Leu	Val	Gln	Leu	Ser	Ala	Val	Asp	Arg	Thr	Leu	
				530					535					540			
15	TCG	TAT	TTT	AAC	GCC	TCA	GAA	CGG	TCG	TCT	CCC	TTT	GTT	GAA	GAA	TTG	1799
	Ser	Tyr	Phe	Asn	Ala	Ser	Glu	Arg	Ser	Ser	Pro	Phe	Val	Glu	Glu	Leu	
			545					550					555				
20	CGT	CAG	ACA	CGC	CGG	GAC	ATC	TCC	AAG	TTT	TTA	GTG	GAC	CCT	GCG	AAT	1847
	Arg	Gln	Thr	Arg	Arg	Asp	Ile	Ser	Lys	Phe	Leu	Val	Asp	Pro	Ala	Asn	
		560					565					570					
	GGG	TTT	ATC	AAC	GGC	AAG	TAC	AAT	TAT	ATT	GTT	GAG	ACA	CCC	ATG	ATT	1895
	Gly	Phe	Ile	Asn	Gly	Lys	Tyr	Asn	Tyr	Ile	Val	Glu	Thr	Pro	Met	Ile	
	575					580				585					590		
25	GCC	GAC	ACA	TTG	AGA	TCC	GGA	CTG	GAC	ATA	TCC	ACT	TTA	TTA	GCT	GCG	1943
	Ala	Asp	Thr	Leu	Arg	Ser	Gly	Leu	Asp	Ile	Ser	Thr	Leu	Leu	Ala	Ala	
					595					600					605		
30	AAC	ACC	GTC	CAC	GAT	GCG	CCA	TCT	GCT	TCC	CAT	CTT	CCG	TTC	GAT	ATC	1991
	Asn	Thr	Val	His	Asp	Ala	Pro	Ser	Ala	Ser	His	Leu	Pro	Phe	Asp	Ile	
				610					615					620			
	AAT	GAC	CCT	GCC	GTC	CTG	AAC	ACG	TTG	CAC	CAT	TTG	ATG	TTG	CAC	ATG	2039
	Asn	Asp	Pro	Ala	Val	Leu	Asn	Thr	Leu	His	His	Leu	Met	Leu	His	Met	
			625					630					635				
35	CGT	TCG	ATA	TAC	CCC	ATC	AAC	GAT	AGC	TCC	AAA	AAT	GCA	ACG	GGT	ATT	2087
	Arg	Ser	Ile	Tyr	Pro	Ile	Asn	Asp	Ser	Ser	Lys	Asn	Ala	Thr	Gly	Ile	
		640					645					650					
40	GCC	CTG	GGG	CGG	TAT	CCT	GAG	GAC	GTA	TAT	GAT	GGA	TAT	GGC	GTT	GGC	2135
	Ala	Leu	Gly	Arg	Tyr	Pro	Glu	Asp	Val	Tyr	Asp	Gly	Tyr	Gly	Val	Gly	
	655					660					665				670		
	GAG	GGA	AAT	CCC	TGG	GTC	CTG	GCC	ACG	TGT	GCC	GCT	TCA	ACA	ACG	CTT	2183
	Glu	Gly	Asn	Pro	Trp	Val	Leu	Ala	Thr	Cys	Ala	Ala	Ser	Thr	Thr	Leu	
					675					680					685		
45	TAT	CAG	CTC	ATT	TAC	AGA	CAC	ATC	TCT	GAG	CAG	CAT	GAC	TTG	GTT	GTC	2231
	Tyr	Gln	Leu	Ile	Tyr	Arg	His	Ile	Ser	Glu	Gln	His	Asp	Leu	Val	Val	
				690					695					700			
50	CCA	ATG	AAC	AAC	GAT	TGT	TCG	AAC	GCA	TTT	TGG	AGC	GAG	CTG	GTA	TTC	2279
	Pro	Met	Asn	Asn	Asp	Cys	Ser	Asn	Ala	Phe	Trp	Ser	Glu	Leu	Val	Phe	
			705					710					715				

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TCC AAC CTC ACG ACT TTG GGA AAT GAC GAA GGC TAT TTG ATT TTG GAG 2327
 Ser Asn Leu Thr Thr Leu Gly Asn Asp Glu Gly Tyr Leu Ile Leu Glu
 720 725 730
 5 TTC AAT ACA CCT GCC TTC AAT CAA ACC ATA CAA AAA ATC TTC CAA CTA 2375
 Phe Asn Thr Pro Ala Phe Asn Gln Thr Ile Gln Lys Ile Phe Gln Leu
 735 740 745 750
 GCT GAT TCA TTC TTG GTC AAG CTG AAA GCC CAC GTG GGA ACA GAC GGG 2423
 Ala Asp Ser Phe Leu Val Lys Leu Lys Ala His Val Gly Thr Asp Gly
 755 760 765
 10 GAA CTA AGT GAA CAA TTT AAC AAA TAC ACA GGG TTT ATG CAG GGT GCC 2471
 Glu Leu Ser Glu Gln Phe Asn Lys Tyr Thr Gly Phe Met Gln Gly Ala
 770 775 780
 15 CAA CAC CTT ACC TGG TCC TAT ACT TCA TTC TGG GAT GCC TAT CAA ATA 2519
 Gln His Leu Thr Trp Ser Tyr Thr Ser Phe Trp Asp Ala Tyr Gln Ile
 785 790 795
 AGA CAA GAA GTT TTA CAG AGT TTG TAGACAAAAA AAAATAAAAG AAAAGCGAGA 2573
 Arg Gln Glu Val Leu Gln Ser Leu
 800 805
 AGTATACACA AGTGTATTTT CTAGATATTT ACATCAAATA TATATATATA TACTTATTTA 2633
 CAAAACCTCTG ATATTATAAA TTAATTAGAT AACTATGTCG GAACGTCCAG CCCAACCACG 2693
 25 TTTGCAGTTC TTTTCACTTT CTCATCCTGT GTCAACTTGT TGCCGGATTG TATCTGTCGA 2753
 C 2754

30 (2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 806 amino acids
 (B) TYPE: amino acid
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

40 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

Met Gln Arg Pro Phe Leu Leu Ala Tyr Leu Val Leu Ser Leu Leu Phe
 1 5 10 15
 45 Asn Ser Ala Leu Gly Phe Pro Thr Ala Leu Val Pro Arg Gly Ser Ser
 20 25 30
 Ser Ser Asn Ile Thr Ser Ser Gly Pro Ser Ser Thr Pro Phe Ser Ser
 35 40 45
 50 Ala Thr Glu Ser Phe Ser Thr Gly Thr Thr Val Thr Pro Ser Ser Ser
 50 55 60
 Lys Tyr Pro Gly Ser Lys Thr Glu Thr Ser Val Ser Ser Thr Thr Glu
 65 70 75 80
 55 Thr Thr Ile Val Pro Thr Thr Thr Thr Thr Ser Val Ile Thr Pro Ser

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	85					90					95						
	Thr	Thr	Thr	Ile	Thr	Thr	Thr	Thr	Val	Cys	Ser	Thr	Gly	Thr	Asn	Ser	Ala
				100						105					110		
5	Gly	Glu	Thr	Thr	Ser	Gly	Cys	Ser	Pro	Lys	Thr	Ile	Thr	Thr	Thr	Val	
			115					120					125				
	Pro	Cys	Ser	Thr	Ser	Pro	Ser	Glu	Thr	Ala	Ser	Glu	Ser	Thr	Thr	Thr	
10		130					135					140					
	Ser	Pro	Thr	Thr	Pro	Val	Thr	Thr	Val	Val	Ala	Thr	Thr	Val	Val	Thr	
	145					150					155						160
	Thr	Glu	Tyr	Ser	Thr	Ser	Thr	Lys	Gln	Gly	Gly	Glu	Ile	Thr	Thr	Thr	
15					165					170							175
	Phe	Val	Thr	Lys	Asn	Ser	Pro	Thr	Thr	Tyr	Leu	Thr	Thr	Ile	Ala	Pro	
				180					185					190			
	Thr	Ser	Ser	Val	Thr	Thr	Val	Thr	Asn	Phe	Thr	Pro	Thr	Thr	Ile	Thr	
20			195					200					205				
	Thr	Thr	Val	Cys	Ser	Thr	Gly	Thr	Asn	Ser	Ala	Gly	Glu	Thr	Thr	Ser	
		210					215					220					
25	Gly	Cys	Ser	Pro	Lys	Thr	Val	Thr	Thr	Thr	Val	Leu	Cys	Ser	Thr	Gly	
	225					230					235						240
	Thr	Gly	Glu	Tyr	Thr	Thr	Glu	Ala	Thr	Ala	Pro	Val	Thr	Thr	Ala	Val	
					245					250							255
30	Thr	Thr	Thr	Val	Val	Thr	Thr	Glu	Ser	Ser	Thr	Gly	Thr	Asn	Ser	Val	
				260					265					270			
	Gly	Lys	Thr	Thr	Thr	Ser	Tyr	Thr	Thr	Lys	Ser	Val	Pro	Thr	Thr	Tyr	
			275					280					285				
35	Val	Phe	Asp	Phe	Gly	Lys	Gly	Ile	Leu	Asp	Gln	Ser	Cys	Gly	Gly	Val	
		290					295					300					
	Phe	Ser	Asn	Asn	Gly	Ser	Ser	Gln	Val	Gln	Leu	Arg	Asp	Val	Val	Leu	
40		305					310					315					320
	Met	Asn	Gly	Thr	Val	Val	Tyr	Asp	Ser	Asn	Gly	Ala	Trp	Asp	Ser	Ser	
					325					330					335		
	Ala	Leu	Glu	Glu	Trp	Leu	Gln	Arg	Gln	Lys	Lys	Val	Ser	Ile	Glu	Arg	
45				340					345					350			
	Ile	Phe	Glu	Asn	Ile	Gly	Pro	Ser	Ala	Val	Tyr	Pro	Ser	Ile	Leu	Pro	
			355					360					365				
50	Gly	Val	Val	Ile	Ala	Ser	Pro	Ser	Gln	Thr	His	Pro	Asp	Tyr	Phe	Tyr	
		370					375					380					
	Gln	Trp	Ile	Arg	Asp	Ser	Ala	Leu	Thr	Ile	Asn	Ser	Ile	Val	Ser	His	
		385					390					395					400
55	Ser	Ala	Asp	Pro	Ala	Ile	Glu	Thr	Leu	Leu	Gln	Tyr	Leu	Asn	Val	Ser	

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	405	410	415
5	Phe His Leu Gln Arg Thr Asn Asn Thr Leu Gly Ala Gly Ile Gly Tyr 420 425 430		
	Thr Asn Asp Thr Val Ala Leu Gly Asp Pro Lys Trp Asn Val Asp Asn 435 440 445		
10	Thr Ala Phe Thr Glu Pro Trp Gly Arg Pro Gln Asn Asp Gly Pro Ala 450 455 460		
	Leu Arg Ser Ile Ala Ile Leu Lys Ile Ile Asp Tyr Ile Lys Gln Ser 465 470 475 480		
15	Gly Thr Asp Leu Gly Ala Lys Tyr Pro Phe Gln Ser Thr Ala Asp Ile 485 490 495		
	Phe Asp Asp Ile Val Arg Trp Tyr Leu Arg Phe Ile Ile Asp His Trp 500 505 510		
20	Asn Ser Ser Gly Phe Asp Leu Trp Glu Glu Val Asn Gly Met His Phe 515 520 525		
	Phe Thr Leu Leu Val Gln Leu Ser Ala Val Asp Arg Thr Leu Ser Tyr 530 535 540		
25	Phe Asn Ala Ser Glu Arg Ser Ser Pro Phe Val Glu Glu Leu Arg Gln 545 550 555 560		
	Thr Arg Arg Asp Ile Ser Lys Phe Leu Val Asp Pro Ala Asn Gly Phe 565 570 575		
30	Ile Asn Gly Lys Tyr Asn Tyr Ile Val Glu Thr Pro Met Ile Ala Asp 580 585 590		
	Thr Leu Arg Ser Gly Leu Asp Ile Ser Thr Leu Leu Ala Ala Asn Thr 595 600 605		
35	Val His Asp Ala Pro Ser Ala Ser His Leu Pro Phe Asp Ile Asn Asp 610 615 620		
	Pro Ala Val Leu Asn Thr Leu His His Leu Met Leu His Met Arg Ser 625 630 635 640		
40	Ile Tyr Pro Ile Asn Asp Ser Ser Lys Asn Ala Thr Gly Ile Ala Leu 645 650 655		
	Gly Arg Tyr Pro Glu Asp Val Tyr Asp Gly Tyr Gly Val Gly Glu Gly 660 665 670		
45	Asn Pro Trp Val Leu Ala Thr Cys Ala Ala Ser Thr Thr Leu Tyr Gln 675 680 685		
	Leu Ile Tyr Arg His Ile Ser Glu Gln His Asp Leu Val Val Pro Met 690 695 700		
50	Asn Asn Asp Cys Ser Asn Ala Phe Trp Ser Glu Leu Val Phe Ser Asn 705 710 715 720		
55	Leu Thr Thr Leu Gly Asn Asp Glu Gly Tyr Leu Ile Leu Glu Phe Asn		

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[illegible]

(2) INFORMATION FOR SEQ ID NO:12:

20 (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 54 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: N

(iv) ANTI-SENSE: N

(ix) FEATURE:

- (A) NAME/KEY: -
(B) LOCATION: 1..54
(D) OTHER INFORMATION: /label= linker
/note= "linker used to create pAYE274"

40 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

ACGGCCCCCC CGGCCACAAA CACAAATATT GATAATATAA AGATGAAGTG GGTA

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(2) INFORMATION FOR SEQ ID NO:13:

45 (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 60 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

55 (ix) FEATURE:

- (A) NAME/KEY: -
(B) LOCATION: 1..60

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(D) OTHER INFORMATION: /label= Linker
/note= "Synthetic oligonucleotide linker used to construct pAYE309"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

5 AGCTTTATTT CCCTTCTTTT TCTCTTTAGC TCGGCTTATT CCAGGAGCTT GGATAAAAGA 60

(2) INFORMATION FOR SEQ ID NO:14:

10 (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 29 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
15 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

20 (iii) HYPOTHETICAL: N

(iv) ANTI-SENSE: N

(ix) FEATURE:

25 (A) NAME/KEY: misc_feature
(B) LOCATION: 1..19
(D) OTHER INFORMATION: /function= "Linker"
/note= "Linker used in construction of pDXL200. Contains SfiI, BglII and HindIII sites."

30 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

GTACGGCCCC CCCGGCCAGA TCTAAGCTT 29

35 **Claims**

Claims for the following Contracting States : DE, FR, IT, NL, SE, CH, BE, AT, LU, GR, DK

40 1. A promoter consisting of the wild-type *Saccharomyces cerevisiae* glycerol-3-phosphate dehydrogenase promoter having the nucleotide sequence defined herein as SEQ1 in isolation from the coding sequence which would normally neighbour the said promoter in wild-type *Saccharomyces cerevisiae*, or a variant or a functional portion of said promoter, wherein said variant or functional portion

45 (i) has at least 80% sequence homology with a region of SEQ1 which region is more homologous than any other region of SEQ1 to the variant or functional portion,
(ii) is at least 100 nucleotides long, and
50 (iii) either retains at least 80% of the ability of said wild-type promoter to promote transcription of a nucleotide sequence encoding a naturally occurring human serum albumin positioned downstream thereof or (a) retains at least 10% of the ability of said wild-type promoter to promote transcription of a nucleotide sequence encoding a naturally occurring human serum albumin positioned downstream thereof and (b) is repressed by complex carbon sources and derepressed by the absence of such sources.

55 2. A promoter according to Claim 1 being at least 200 nucleotides long.

3. A cloning vector or a yeast expression vector comprising a promoter according to any one of the preceding claims adjacent a restriction site such that a heterologous coding sequence may be located downstream of the promoter

and in the correct reading frame in relation to a translational start codon.

4. A yeast expression vector according to Claim 3 comprising a heterologous coding sequence inserted as said.
5. A yeast expression vector according to Claim 4 wherein the heterologous coding sequence encodes human serum albumin or a variant or part thereof, optionally with a secretion leader sequence.
6. A yeast expression vector according to Claim 4 wherein the heterologous coding sequence encodes the glucoamylase of *S. cerevisiae* var *diastaticus*.
7. A yeast transformed with an expression vector according to Claim 4, 5 or 6.
8. A process for preparing a polypeptide, comprising fermenting a yeast according to Claim 7 and at least partially purifying the polypeptide expressed by the said heterologous coding sequence.
9. A process according to Claim 8 wherein the yeast is initially grown on a carbon source or sources which repress expression of the polypeptide and subsequently the carbon source is changed to a non-repressing compound or mixture of such compounds.

Claims for the following Contracting State : ES

1. A process for preparing a promoter by conventional polynucleotide manipulation techniques, characterized in that the promoter consists of the wild-type *Saccharomyces cerevisiae* glycerol-3-phosphate dehydrogenase promoter having the nucleotide sequence defined herein as SEQ1 in isolation from the coding sequence which would normally neighbour the said promoter in wild-type *Saccharomyces cerevisiae*, or a variant or a functional portion of said promoter, wherein said variant or functional portion
 - (i) has at least 80% sequence homology with a region of SEQ1 which region is more homologous than any other region of SEQ1 to the variant or functional portion,
 - (ii) is at least 100 nucleotides long, and
 - (iii) either retains at least 80% of the ability of said wild-type promoter to promote transcription of a nucleotide sequence encoding a naturally occurring human serum albumin positioned downstream thereof or (a) retains at least 10% of the ability of said wild-type promoter to promote transcription of a nucleotide sequence encoding a naturally occurring human serum albumin positioned downstream thereof and (b) is repressed by complex carbon sources and derepressed by the absence of such sources.
2. A process according to Claim 1 wherein the promoter is at least 200 nucleotides long.
3. A process for preparing a cloning vector or a yeast expression vector comprising a promoter prepared according to any one of the preceding claims wherein the promoter is placed adjacent a restriction site such that a heterologous coding sequence may be located downstream of the promoter and in the correct reading frame in relation to a translational start codon.
4. A process according to Claim 3 additionally comprising inserting a heterologous coding sequence inserted as said.
5. A process according to Claim 4 wherein the heterologous coding sequence encodes human serum albumin or a variant or part thereof, optionally with a secretion leader sequence.
6. A process according to Claim 4 wherein the heterologous coding sequence encodes the glucoamylase of *S. cerevisiae* var *diastaticus*.
7. A process of transforming a yeast characterised in that the yeast is transformed with an expression vector according to Claim 4, 5 or 6.
8. A process for preparing a polypeptide, comprising fermenting a yeast prepared according to Claim 7 and at least partially purifying the polypeptide expressed by the said heterologous coding sequence.

9. A process according to Claim 8 wherein the yeast is initially grown on a carbon source or sources which repress expression of the polypeptide and subsequently the carbon source is changed to a non-repressing compound or mixture of such compounds.

Patentansprüche

Patentansprüche für folgende Vertragsstaaten : DE, FR, IT, NL, SE, CH, BE, AT, LU, GR, DK

1. Promotor, bestehend aus dem Wildtyp *Saccharomyces cerevisiae* Glycerin-3-phosphat-Dehydrogenase-Promotor mit der hierin als SEQ1 definierten Nucleotidsequenz isoliert von der Codiersequenz, die normalerweise dem Promotor im Wildtyp *Saccharomyces cerevisiae* benachbart wäre, oder einer Variante oder einem funktionellen Teil dieses Promotors, wobei die Variante oder der funktionelle Teil
 - (i) eine mindestens 80%ige Sequenzhomologie mit einem SEQ1-Bereich, der zu der Variante oder dem funktionellen Teil stärker homolog ist als irgendein anderer SEQ1-Bereich, aufweist;
 - (ii) mindestens 100 Nucleotide lang ist und
 - (iii) entweder mindestens 80% der Fähigkeit des Wildtyp-Promotors zur Promotion der Transkription einer Nucleotidsequenz mit Codierung für ein natürlich vorkommendes Humanserumalbumin in einer Position stromabwärts desselben behält oder (a) mindestens 10% der Fähigkeit des Wildtyp-Promotors zur Promotion der Transkription einer Nucleotidsequenz mit Codierung für ein natürlich vorkommendes Humanserumalbumin in einer Position stromabwärts desselben behält und (b) durch komplexe Kohlenstofflieferanten einer Repression unterliegt und bei Abwesenheit solcher Lieferanten von der Repression befreit ist.
2. Promotor nach Anspruch 1, der mindestens 200 Nucleotide lang ist.
3. Klonierungsvektor oder Hefeexpressionsvektor, umfassend einen Promotor nach einem der vorhergehenden Ansprüche in Nachbarschaft zu einer Restriktionsstelle dergestalt, daß stromabwärts vom Promotor und im korrekten Leserahmen in bezug auf ein Translationsstartcodon eine heterologe Codiersequenz angeordnet sein kann.
4. Hefeexpressionsvektor nach Anspruch 3, umfassend eine - wie dargelegt - insertierte bzw. eingefügte heterologe Codiersequenz.
5. Hefeexpressionsvektor nach Anspruch 4, wobei die heterologe Codiersequenz für Humanserumalbumin oder eine Variante oder einen Teil desselben, gegebenenfalls mit einer Sekretionsleadersequenz, kodiert.
6. Hefeexpressionsvektor nach Anspruch 4, wobei die heterologe Codiersequenz für die Glucoamylase von *S. cerevisiae* var. *diastaticus* codiert.
7. Hefe, welche mit einem Expressionsvektor nach Anspruch 4, 5 oder 6 transformiert ist.
8. Verfahren zur Herstellung eines Polypeptids durch Fermentieren einer Hefe nach Anspruch 7 und zumindest Teilreinigen des durch die heterologe Codiersequenz exprimierten Polypeptids.
9. Verfahren nach Anspruch 8, wobei die Hefe zunächst auf (einem) die Expression des Polypeptids unterdrückenden Kohlenstofflieferanten wachengelassen wird und danach der Kohlenstofflieferant gegen eine nicht-unterdrückende Verbindung oder ein Gemisch solcher Verbindungen ausgetauscht wird.

Patentansprüche für folgenden Vertragsstaat : ES

1. Verfahren zur Herstellung eines Promotors durch übliche Polynucleotidmanipulationsmaßnahmen, dadurch gekennzeichnet, daß der Promotor aus dem Wildtyp *Saccharomyces cerevisiae* Glycerin-3-phosphat-Dehydrogenase-Promotor mit der hierin als SEQ1 definierten Nucleotidsequenz isoliert von der Codiersequenz, die normalerweise dem Promotor im Wildtyp *Saccharomyces cerevisiae* benachbart wäre, oder einer Variante oder einem funktionellen Teil dieses Promotors, wobei die Variante oder der funktionelle Teil

- (i) eine mindestens 80%ige Sequenzhomologie mit einem SEQ1-Bereich, der zu der Variante oder dem funktionellen Teil stärker homolog ist als irgendein anderer SEQ1-Bereich, aufweist;
- (ii) mindestens 100 Nucleotide lang ist und
- (iii) entweder mindestens 80% der Fähigkeit des Wildtyp-Promotors zur Promotion der Transkription einer Nucleotidsequenz mit Codierung für ein natürlich vorkommendes Humanserumalbumin in einer Position stromabwärts desselben behält oder (a) mindestens 10% der Fähigkeit des Wildtyp-Promotors zur Promotion der Transkription einer Nucleotidsequenz mit Codierung für ein natürlich vorkommendes Humanserumalbumin in einer Position stromabwärts desselben behält und (b) durch komplexe Kohlenstofflieferanten einer Repression unterliegt und bei Abwesenheit solcher Lieferanten von der Repression befreit ist,

besteht.

2. Verfahren nach Anspruch 1, wobei der Promotor mindestens 200 Nucleotide lang ist.
3. Verfahren zur Herstellung eines Klonierungsvektors oder eines Hefeexpressionsvektors, umfassend einen nach einem der vorhergehenden Ansprüche hergestellten Promotor, wobei der Promotor in Nachbarschaft zu einer Restriktionsstelle plaziert ist, dergestalt, daß stromabwärts vom Promotor und im korrekten Leserahmen in bezug auf ein Translationsstartcodon eine heterologe Codiersequenz angeordnet sein kann.
4. Verfahren nach Anspruch 3, bei welchem zusätzlich - wie dargelegt - eine heterologe Codiersequenz insertiert bzw. eingefügt wird.
5. Verfahren nach Anspruch 4, wobei die heterologe Codiersequenz für Humanserumalbumin oder eine Variante oder einen Teil desselben, gegebenenfalls mit einer Sekretionsleadersequenz, kodiert.
6. Verfahren nach Anspruch 4, wobei die heterologe Codiersequenz für die Glucoamylase von *S. cerevisiae* var. diastaticus codiert.
7. Verfahren zur Transformation einer Hefe, dadurch gekennzeichnet, daß die Hefe mit einem Expressionsvektor nach Anspruch 4, 5 oder 6 transformiert wird.
8. Verfahren zur Herstellung eines Polypeptids durch Fermentieren einer nach Anspruch 7 hergestellten Hefe und zumindestens Teilreinigen des durch die heterologe Codiersequenz exprimierten Polypeptids.
9. Verfahren nach Anspruch 8, wobei die Hefe zunächst auf (einem) die Expression des Polypeptids unterdrückenden Kohlenstofflieferanten wachengelassen wird und danach der Kohlenstoff lieferant gegen eine nicht-unterdrückende Verbindung oder ein Gemisch solcher Verbindungen ausgetauscht wird.

Revendications

Revendications pour les Etats contractants : DE, FR, IT, NL, SE, CH, BE, AT, LU, GR, DK

1. Promoteur consistant en le promoteur de la glycérol-3-phosphate déshydrogénase de *Saccharomyces cerevisiae* de type sauvage ayant la séquence de nucléotides définie ici en tant que SEQ1 isolée de la séquence codante qui est normalement voisine dudit promoteur dans *Saccharomyces cerevisiae* de type sauvage, ou une variante ou une partie fonctionnelle dudit promoteur, ladite variante ou partie fonctionnelle :
 - (i) ayant au moins 80 % d'homologie de séquence avec une région de SEQ1, laquelle région est plus homologue à la variante ou à la partie fonctionnelle qu'une quelconque autre région de SEQ1,
 - (ii) étant longue d'au moins 100 nucléotides, et
 - (iii) retenant au moins 80 % de la capacité dudit promoteur de type sauvage à favoriser la transcription d'une séquence de nucléotides codant pour une séralbumine humaine naturelle positionnée en aval de celui-ci ou (a) retenant au moins 10 % de la capacité dudit promoteur de type sauvage à favoriser la transcription d'une séquence de nucléotides codant pour une séralbumine humaine naturelle positionnée en aval de celui-ci et (b) étant réprimée par des sources de carbone complexes et déréprimée par l'absence de telles sources.

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2. Promoteur selon la revendication 1, long d'au moins 200 nucléotides.
3. Vecteur de clonage ou vecteur d'expression pour levure, comprenant un promoteur selon l'une quelconque des revendications précédentes, adjacent à un site de restriction de sorte qu'une séquence codante hétérologue puisse être située en aval du promoteur et dans le cadre de lecture correct par rapport à un codon d'initiation de traduction.
4. Vecteur d'expression pour levure selon la revendication 3, comprenant une séquence codante hétérologue insérée comme décrit.
5. Vecteur d'expression pour levure selon la revendication 4, dans lequel la séquence codante hétérologue code pour la sérumalbumine humaine ou une variante ou partie de celle-ci, éventuellement avec une séquence leader de sécrétion.
6. Vecteur d'expression pour levure selon la revendication 4, dans lequel la séquence codante hétérologue code pour la glucoamylase de *S. cerevisiae* var *diastaticus*.
7. Levure transformée avec un vecteur d'expression selon la revendication 4, 5 ou 6.
8. Procédé pour préparer un polypeptide, comprenant la fermentation d'une levure selon la revendication 7 et la purification au moins partielle du polypeptide exprimé par ladite séquence codante hétérologue.
9. Procédé selon la revendication 8, dans lequel la levure est initialement développée sur une ou des source(s) de carbone qui réprime(nt) l'expression du polypeptide, après quoi la source de carbone est changée par un composé non répresseur ou un mélange de tels composés.

Revendications pour l'Etat contractant : ES

1. Procédé de préparation d'un promoteur par des techniques de manipulation de polynucléotides conventionnelles, caractérisé en ce que le promoteur est constitué du promoteur glycérol-3-phosphate déshydrogénase de *Saccharomyces cerevisiae* de type sauvage ayant la séquence de nucléotides définie ici en tant que SEQ1 isolée de la séquence codante qui est normalement voisine dudit promoteur dans *Saccharomyces cerevisiae* de type sauvage, ou une variante ou une partie fonctionnelle dudit promoteur, ladite variante ou partie fonctionnelle :
 - (i) ayant au moins 80 % d'homologie de séquence avec une région de SEQ1, laquelle région est plus homologue à la variante ou à la partie fonctionnelle qu'une quelconque autre région de SEQ1,
 - (ii) étant longue d'au moins 100 nucléotides, et
 - (iii) retenant au moins 80 % de la capacité dudit promoteur de type sauvage à favoriser la transcription d'une séquence de nucléotides codant pour une sérumalbumine humaine naturelle positionnée en aval de celui-ci ou (a) retenant au moins 10 % de la capacité dudit promoteur de type sauvage à favoriser la transcription d'une séquence de nucléotides codant pour une sérumalbumine humaine naturelle positionnée en aval de celui-ci et (b) étant réprimée par des sources de carbone complexes et déréprimée par l'absence de telles sources.
2. Procédé selon la revendication 1, dans lequel le promoteur est long d'au moins 200 nucléotides.
3. Procédé pour préparer un vecteur de clonage ou un vecteur d'expression pour levure, comprenant un promoteur préparé selon l'une quelconque des revendications précédentes, dans lequel le promoteur est placé adjacent à un site de restriction de sorte qu'une séquence codante hétérologue puisse être située en aval du promoteur et dans le cadre de lecture correct par rapport à un codon d'initiation de traduction.
4. Procédé selon la revendication 3, comprenant en plus l'insertion d'une séquence codante hétérologue insérée comme décrit.
5. Procédé selon la revendication 4, dans lequel la séquence codante hétérologue code pour la sérumalbumine humaine ou une variante ou partie de celle-ci, éventuellement avec une séquence leader de sécrétion.
6. Procédé selon la revendication 4, dans lequel la séquence codante hétérologue code pour la glucoamylase de *S.*

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cerevisiae var *diastaticus*.

7. Procédé pour transformer une levure, caractérisé en ce que la levure est transformée avec un vecteur d'expression selon la revendication 4, 5 ou 6.

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8. Procédé pour préparer un polypeptide, comprenant la fermentation d'une levure selon la revendication 7 et la purification au moins partielle du polypeptide exprimé par ladite séquence codante hétérologue.

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9. Procédé selon la revendication 8, dans lequel la levure est initialement développée sur une ou des source(s) de carbone qui réprime(nt) l'expression du polypeptide, après quoi la source de carbone est changée par un composé non répresseur ou un mélange de tels composés.

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Figure 1

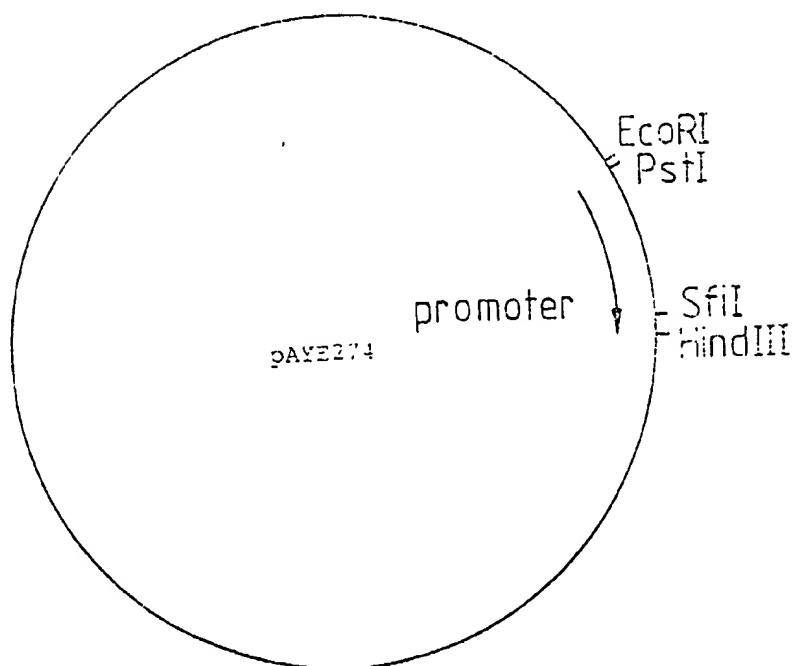
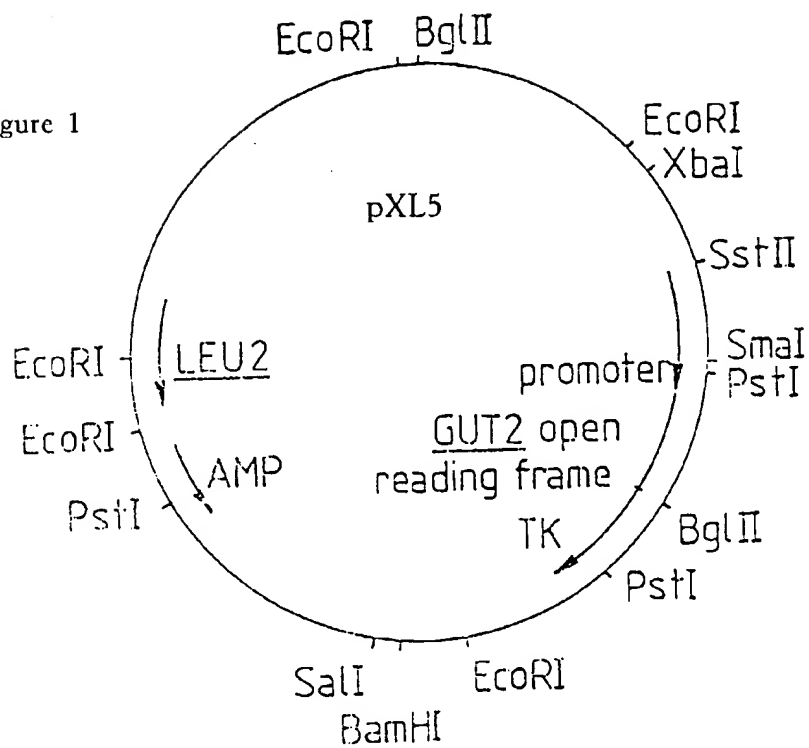


FIGURE 2

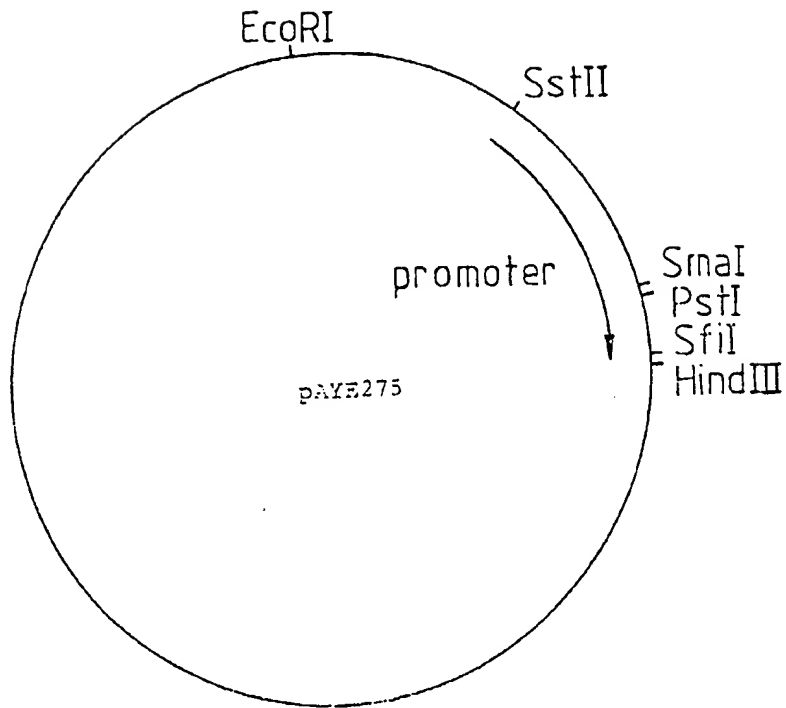


FIGURE 3

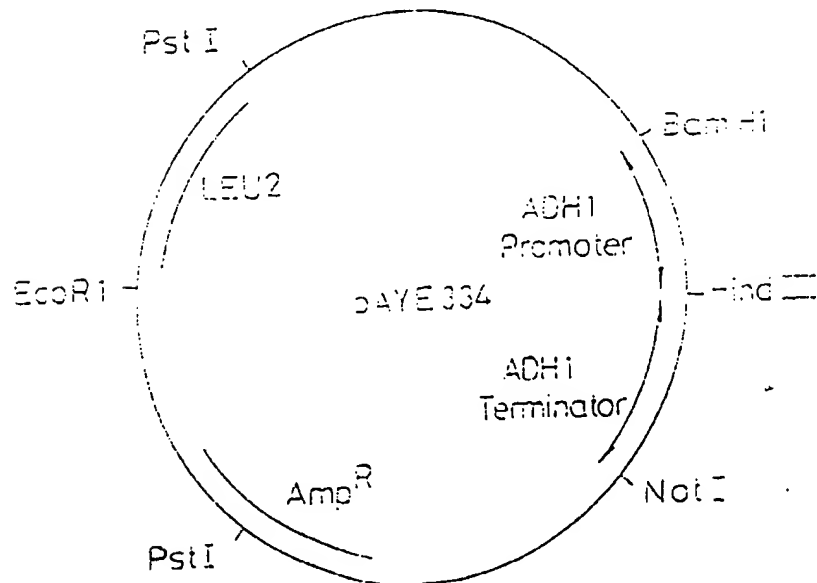


Fig. 4

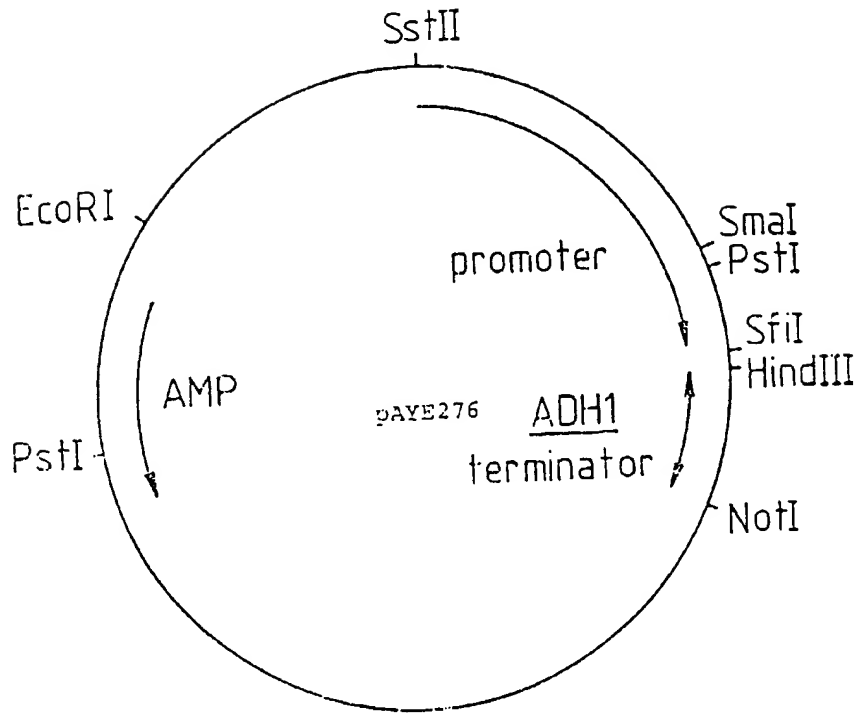


FIGURE 5

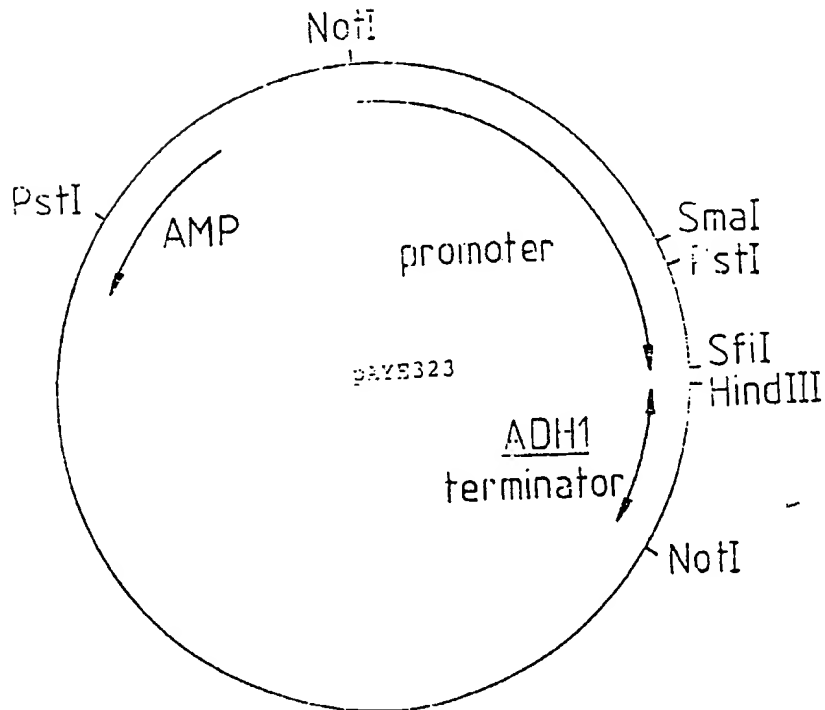


FIGURE 6

FIGURE 7

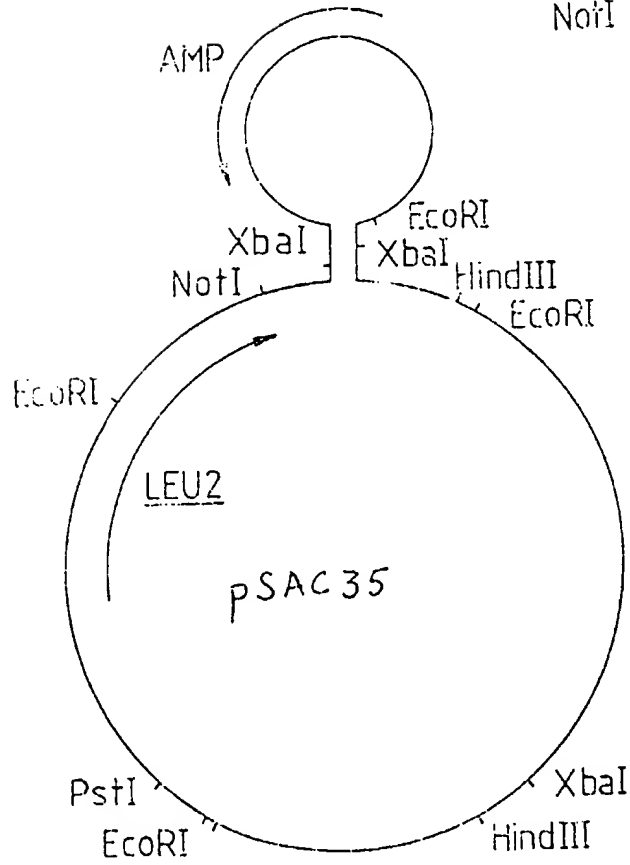
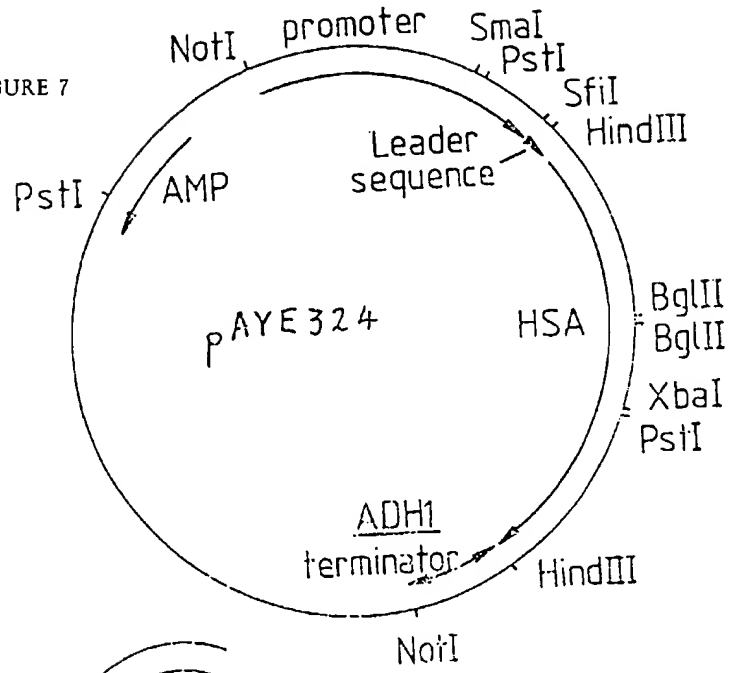


FIGURE 8

FIGURE 9

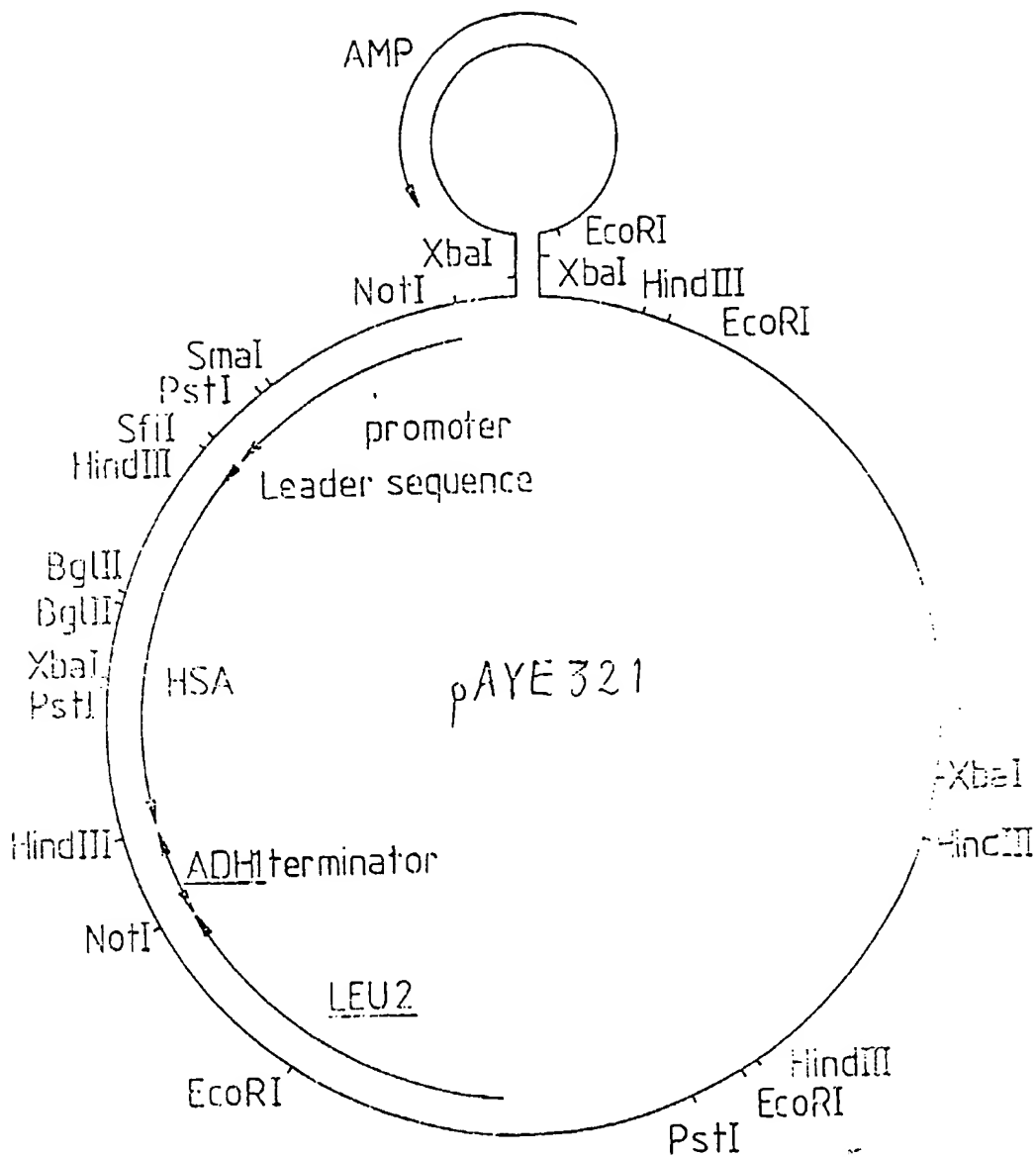


Figure 10

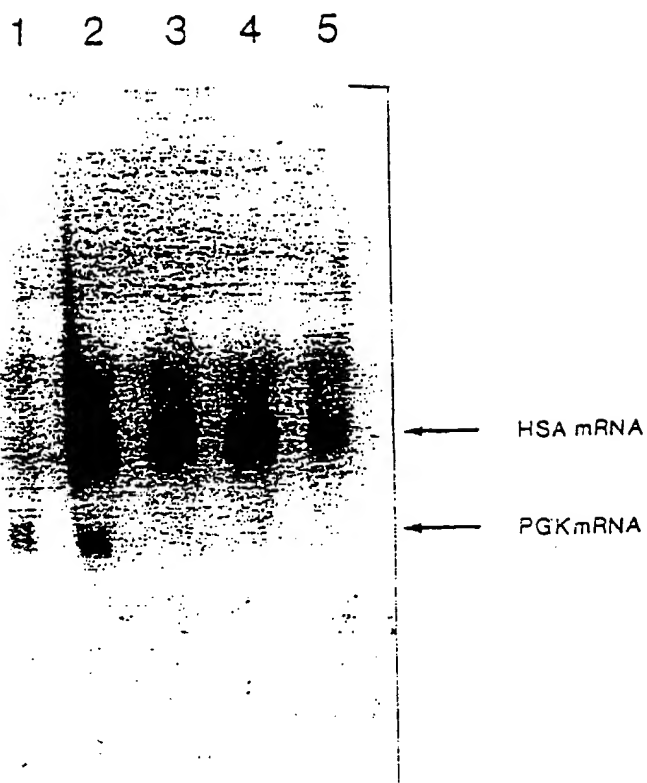


Figure 11

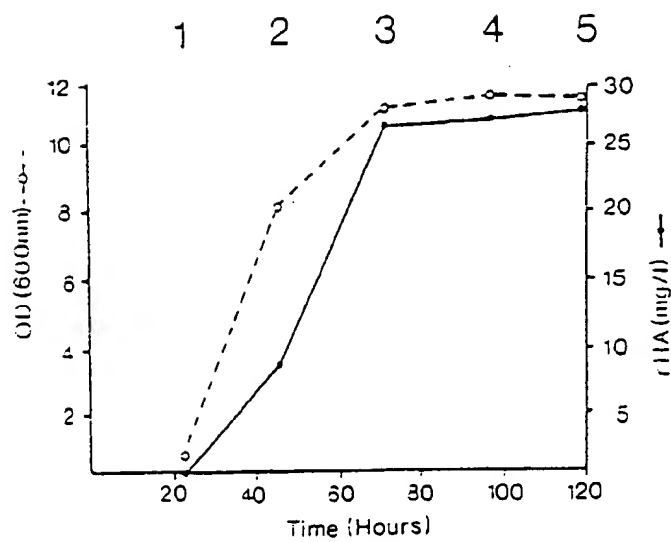


Figure 12

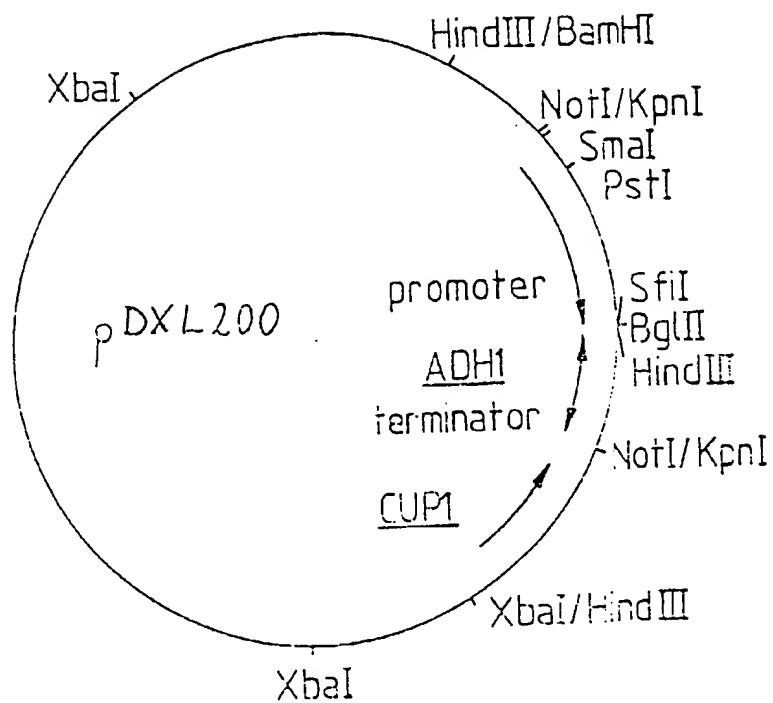


Figure 13

